

# SPECIFICATION

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## **NOVEL FLEA ECDYSONE AND ULTRASPIRACLE NUCLEIC ACID MOLECULES, PROTEINS AND USES THEREOF**

### **Cross Reference to Related Applications**

The present application is a Divisional of U.S. Serial No. 09/435,019, filed November 5, 1999, which claims priority to U.S. Provisional Patent Application Serial No. 60/107,559, filed November 6, 1998, each entitled "NOVEL FLEA ECDYSONE AND ULTRASPIRACLE NUCLEIC ACID MOLECULES, PROTEINS AND USES THEREOF".

### **Field of the Invention**

[0001] The present invention relates to flea ecdysone and ultraspiracle nucleic acid molecules, proteins encoded by such nucleic acid molecules, antibodies raised against such proteins, and inhibitors of such proteins. The present invention also includes therapeutic compositions comprising such nucleic acid molecules, proteins, antibodies, and/or other inhibitors, as well as their use to protect an animal from flea infestation.

### **Background of the Invention**

[0002] Flea infestation of animals is a health and economic concern because fleas are known to cause and/or transmit a variety of diseases. Fleas directly cause a variety of diseases, including allergies, and also carry a variety of infectious agents including, but not limited to, endoparasites (e.g., nematodes, cestodes, trematodes and protozoa), bacteria and viruses. In particular, the bites of fleas are a problem for animals maintained as pets because the infestation becomes a source of annoyance not only for the pet but also for the pet owner who may find his or her home generally

contaminated with insects. As such, fleas are a problem not only when they are on an animal but also when they are in the general environment of the animal.

[0003] Bites from fleas are a particular problem because they not only can lead to disease transmission but also can cause a hypersensitive response in animals which is manifested as disease. For example, bites from fleas can cause an allergic disease called flea allergic (or allergy) dermatitis (FAD). A hypersensitive response in animals typically results in localized tissue inflammation and damage, causing substantial discomfort to the animal.

[0004] The medical importance of flea infestation has prompted the development of reagents capable of controlling flea infestation. Commonly encountered methods to control flea infestation are generally focused on use of insecticides. While some of these products are efficacious, most, at best, offer protection of a very limited duration. Furthermore, many of the methods are often not successful in reducing flea populations. In particular, insecticides have been used to prevent flea infestation of animals by adding such insecticides to shampoos, powders, collars, sprays, foggers and liquid bath treatments (i.e., dips). Reduction of flea infestation on the pet has been unsuccessful for one or more of the following reasons: (1) failure of owner compliance (frequent administration is required); (2) behavioral or physiological intolerance of the pet to the pesticide product or means of administration; and (3) the emergence of flea populations resistant to the prescribed dose of pesticide. Flea populations, however, have been found to become resistant to insecticides.

[0005] 20-Hydroxyecdysone (ecdysone) is the insect steroid hormone which regulates molting and metamorphosis. The ability of ecdysone to have a pleiotropic effect upon various tissues is dependent upon the formation of a complex of ecdysone with its receptor (EcR) and its heterodimeric partner, ultraspiracle (USP). This complex then binds to ecdysone response elements (EcRE) found within the promoters of insect genes, and thereby affecting DNA transcription. EcR by itself has been reported to be incapable of high affinity binding or transcriptional activation, rather, these activities appear to be dependent upon heterodimer formation with USP, Yao et al., 1993, Nature 366, 476-479.

[0006] Prior investigators have described certain insect EcR protein or nucleic acid sequences, including for example, *Bombyx mori*, Swevers et al., 1995, *Insect Biochem. Mol. Biol.* 25(7), 857-866; *Drosophila melanogaster*, Koelle et al., 1991, *Cell* 67(1), 59 and *Manduca sexta*, Fujiwara et al., 1995, *Insect Biochem. Mol. Biol.* 25(7), 845 and certain insect USP protein and nucleic acid sequences, including for example, *Bombyx mori*, Tzertzinis et al., 1994, *J. Mol. Biol.* 238, 479-486; *Drosophila melanogaster*, Oro et al., 1990, *Nature*, 347(6290) 298-301; and *Manduca sexta*, Jindra et al., GenBank Accession 1718061. Prior investigators have also described mammalian homologs of EcR and USP, Giguere et al., 1987, *Nature* 330(6149), 624-629; Cooke et al., 1996, GenBank Accession 1350913; Leid et al., 1992, *Cell* 68(2), 377-395; and amphibian homologs, Blumberg et al., 1992, *Proc. Natl. Acad. Sci., U.S.A.* 89(6), 2321-2325.

[0007] Identification of flea EcR and USP of the present invention is surprising, however, due to the source from which these molecules were identified. Most lepidopterans and dipterans are better characterized, relative to *C. felis*, with respect to visible signs of molting, the only stages which should possess high levels of ecdysone. Ecdysone is necessary for the up regulation of mRNA encoding EcR and USP. Therefore, the lack of clear, easily visible signs of molting in *C. felis* make the likelihood of finding cDNA containing EcR or USP message in the larval and prepupal cDNA unexpected.

[0008] Thus, there remains a need to develop a reagent and a method to protect animals from flea infestation.

## Summary of Invention

[0009] The present invention relates to a novel product and process for protection of animals from flea infestation. Identification of flea EcR and USP of the present invention is surprising, however, due to the source from which these molecules were identified. Most lepidopterans and dipterans are better characterized, relative to *C. felis*, with respect to visible signs of molting, the only stages which should possess high levels of ecdysone. Ecdysone is necessary for the up regulation of mRNA encoding EcR and USP. Therefore, the lack of clear, easily visible signs of molting in *C. felis* make the likelihood of finding cDNA containing EcR or USP message in the larval and prepupal cDNA unexpected.

[0010] According to the present invention there are provided flea ecdysone receptor (EcR) or ultraspiracle (USP) proteins, and mimetopes thereof; flea EcR and USP nucleic acid molecules, including those that encode such proteins; antibodies raised against such EcR and USP proteins (i.e., anti-flea EcR and USP antibodies); and compounds that inhibit flea EcR and USP activity (i.e., inhibitory compounds or inhibitors).

[0011] The present invention also includes methods to obtain such proteins, mimetopes, nucleic acid molecules, antibodies and inhibitory compounds. Also included in the present invention are therapeutic compositions comprising a protective compound derived from a protein of the present invention that inhibits the binding between ecdysone receptor and ecdysone.

[0012] One embodiment of the present invention is an isolated nucleic acid molecule having at least about 34 nucleotides which hybridizes with a nucleic acid sequence having SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16 and/or SEQ ID NO:18 under conditions that allow about 30% base pair mismatch. Another embodiment of the present invention is an isolated nucleic acid molecule having at least about 30 nucleotides which hybridizes with a nucleic acid sequence having SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29; SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35 and/or SEQ ID NO:37 under conditions that allow about 30% base pair mismatch.

[0013] The present invention also relates to recombinant molecules, recombinant viruses and recombinant cells that include a nucleic acid molecule of the present invention. Also included are methods to produce such nucleic acid molecules, recombinant molecules, recombinant viruses and recombinant cells.

[0014] The present invention also relates to mimetopes of flea EcR and/or USP proteins as well as to isolated antibodies that selectively bind to flea EcR and/or USP proteins or mimetopes thereof. Also included are methods, including recombinant methods, to produce proteins, mimetopes and antibodies of the present invention.

[0015] Another embodiment of the present invention includes an isolated flea ecdysone receptor protein selected from the group consisting of a protein comprising (a) an amino acid sequence that is at least about 70% identical to an amino acid sequence

selected from the group consisting of SEQ ID NO:6 and/or SEQ ID NO:14, wherein said protein is at least about 71 amino acids residues in length; (b) a protein consisting of an amino acid sequence having SEQ ID NO:64 and/or SEQ ID NO:65, and fragments thereof, wherein said protein has at least a portion of an ecdysone receptor DNA binding domain; (c) a protein consisting of an amino acid sequence having SEQ ID NO:66 and/or SEQ ID NO:67, and fragments thereof, wherein said protein has at least a portion of an ecdysone receptor ligand binding domain; or (d) a protein encoded by an allelic variant of nucleic acid molecules encoding any protein of (a), (b), and/or (c).

[0016] Another embodiment of the present invention includes an isolated flea ultraspiracle protein selected from the group consisting of: (a) a protein comprising an amino acid sequence that is at least about 70% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:33, wherein said protein is at least about 72 amino acid residues in length; (b) a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, and fragments thereof, wherein said protein has at least a portion of an ultraspiracle protein that is capable of affecting binding of ecdysone receptor to ecdysone; and (c) a protein encoded by an allelic variant of a nucleic acid molecule which encodes any protein of (a) or (b).

[0017] Another embodiment of the present invention includes an isolated protein encoded by a nucleic acid molecule having a nucleic acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:26, SEQ ID NO:29, SEQ ID NO:32, and/or SEQ ID NO:35.

[0018] Another embodiment of the present invention includes a method to identify a compound capable of inhibiting EcR activity, the method comprising: (a) contacting an isolated flea EcR protein comprising an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:14, with a putative inhibitory compound under conditions in which, in the absence of the putative inhibitory compound, the protein has EcR activity, and (b) determining if the putative inhibitory compound inhibits EcR activity.

[0019] Another embodiment of the present invention includes a method to identify a compound capable of inhibiting flea activity, the method comprising: (a) contacting an

isolated flea USP protein comprising an amino acid sequence consisting of SEQ ID NO:27 and SEQ ID NO:33, with a putative inhibitory compound under conditions in which, in the absence of the putative inhibitory compound, the protein has USP activity, and (b) determining if the putative inhibitory compound inhibits USP activity.

## Detailed Description

[0020] The present invention provides for isolated flea ecdysone (EcR) and ultraspiracle (USP) proteins, isolated flea EcR and USP nucleic acid molecules, isolated antibodies directed against flea EcR and USP proteins, and compounds able to inhibit flea EcR and/or USP function (i.e., inhibitory compounds). As used herein, the terms isolated flea EcR and USP proteins and isolated flea EcR and USP nucleic acid molecules refer to EcR and USP proteins and EcR and USP nucleic acid molecules derived from fleas; as such the proteins and nucleic acid molecules can be isolated from an organism or prepared recombinantly or synthetically. Flea EcR nucleic acid molecules of known length are denoted "nECR<sub>#</sub>", for example nECR<sub>4148</sub>, wherein "#" refers to the number of nucleotides in that molecule, and EcR proteins of known length are denoted "Pecr<sub>#</sub>" (for example Pecr<sub>562</sub>) wherein "#" refers to the number of amino acid residues in that molecule. Similarly, USP nucleic acid molecules and proteins of known length are denoted "nUSP<sub>#</sub>" and "Pusp<sub>#</sub>", respectively. The proteins and nucleic acid molecules of the present invention can be obtained from their natural source, or can be produced using, for example, recombinant nucleic acid technology or chemical synthesis. Also included in the present invention is the use of these proteins, nucleic acid molecules, antibodies, and inhibitory compounds as therapeutic compositions to protect animals from flea infestation as well as in other applications, such as those disclosed below.

[0021]

Flea EcR and USP proteins and nucleic acid molecules of the present invention have utility because they represent novel targets for anti-arthropod vaccines and chemotherapeutic drugs. The products and processes of the present invention are advantageous because they enable the inhibition of arthropod development, metamorphosis, feeding, digestion and reproduction processes that involve EcR and/or USP proteins. While not being bound by theory, it is believed that expression of arthropod EcR and USP proteins are developmentally regulated, thereby suggesting

that EcR and USP proteins are involved in arthropod development and/or reproduction. The present invention is particularly advantageous because the proteins of the present invention were identified in larval fleas, thereby suggesting the importance of the proteins as developmental proteins.

[0022] Tissue can be obtained from unfed fleas or from fleas that recently consumed a blood meal (i.e., blood-fed fleas). Such flea tissues are referred to herein as, respectively, unfed flea and fed flea tissue. Preferred flea tissue from which to obtain an EcR and/or USP formulation of the present invention include, but are not limited to, unfed or fed 1<sup>st</sup> instar larvae; fed 3<sup>rd</sup> instar larvae, fed wandering larvae, fed prepupal larvae, fed pupae and whole unfed or fed adult fleas. Preferred flea tissue from which to obtain an EcR and/or USP formulation of the present invention includes third instar larvae, wandering larvae, prepupal larvae, pupae, and adult fleas.

[0023] In a preferred embodiment, a formulation of the present invention comprises a flea EcR protein comprising amino acid sequence SEQ ID NO:6 or SEQ ID NO:14, and/or a flea USP protein comprising amino acid sequence SEQ ID NO:27 or SEQ ID NO:33.

[0024] One embodiment of the present invention is an isolated protein that includes a flea EcR and/or USP protein. It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, a protein, a nucleic acid molecule, an antibody and a therapeutic composition refers to "one or more" or "at least one" protein, nucleic acid molecule, antibody and therapeutic composition respectively. As such, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably. According to the present invention, an isolated, or biologically pure, protein, is a protein that has been removed from its natural milieu. As such, "isolated" and "biologically pure" do not necessarily reflect the extent to which the protein has been purified. An isolated protein of the present invention can be obtained from its natural source, can be produced using recombinant DNA technology, or can be produced by chemical synthesis.

[0025] As used herein, isolated flea EcR and/or USP proteins of the present invention can be full-length proteins or any homolog of such proteins. An isolated protein of the

present invention, including a homolog, can be identified in a straight-forward manner by the protein's ability to elicit an immune response against a flea EcR or USP protein or by the protein's EcR or USP activity. Examples of flea EcR and USP homolog proteins include flea EcR and USP proteins in which amino acids have been deleted (e.g., a truncated version of the protein, such as a peptide), inserted, inverted, substituted and/or derivatized (e.g., by glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitoylation, amidation and/or addition of glycerophosphatidyl inositol) such that the homolog includes at least one epitope capable of eliciting an immune response against a flea EcR or USP protein, and/or of binding to an antibody directed against a flea EcR or USP protein. That is, when the homolog is administered to an animal as an immunogen, using techniques known to those skilled in the art, the animal will produce an immune response against at least one epitope of a natural flea EcR or USP protein. The ability of a protein to effect an immune response can be measured using techniques known to those skilled in the art. As used herein, the term "epitope" refers to the smallest portion of a protein or other antigen capable of selectively binding to the antigen binding site of an antibody or a T cell receptor. It is well accepted by those skilled in the art that the minimal size of a protein epitope is about four to six amino acids. As is appreciated by those skilled in the art, an epitope can include amino acids that naturally are contiguous to each other as well as amino acids that, due to the tertiary structure of the natural protein, are in sufficiently close proximity to form an epitope. According to the present invention, an epitope includes a portion of a protein comprising at least about 4 amino acids, at least about 5 amino acids, at least about 6 amino acids, at least about 10 amino acids, at least about 15 amino acids, at least about 20 amino acids, at least about 25 amino acids, at least about 30 amino acids, at least about 35 amino acids, at least about 40 amino acids or at least about 50 amino acids in length. In one embodiment of the present invention a flea homolog protein has EcR or USP activity. Examples of methods to detect EcR and/or USP activity are disclosed herein. Flea EcR and USP homolog proteins can be the result of natural allelic variation or natural mutation. Flea EcR and USP protein homologs of the present invention can also be produced using techniques known in the art including, but not limited to, direct modifications to the protein or modifications to the gene encoding the protein using, for example, classic or recombinant DNA techniques to effect random or targeted



mutagenesis.

[0026] Flea EcR and USP proteins of the present invention are encoded by flea EcR and USP nucleic acid molecules, respectively. As used herein, flea EcR and USP nucleic acid molecules include nucleic acid sequences related to natural flea EcR and USP genes, and, preferably, to *Ctenocephalides felis* EcR and USP genes. As used herein, flea EcR and USP genes include all regions such as regulatory regions that control production of flea EcR and USP proteins encoded by such genes (such as, but not limited to, transcription, translation or post-translation control regions) as well as the coding region itself, and any introns or non-translated coding regions. As used herein, a gene that "includes" or "comprises" a sequence may include that sequence in one contiguous array, or may include the sequence as fragmented exons. As used herein, the term "coding region" refers to a continuous linear array of nucleotides that translates into a protein. A full-length coding region is that coding region that is translated into a full-length, i.e., a complete protein as would be initially translated in its natural milieu, prior to any post-translational modifications.

[0027] One embodiment of the present invention is a *C. felis* EcR gene that includes the nucleic acid sequence SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:13, and/or SEQ ID NO:16, as well as the complements of any of these nucleic acid sequences; and a *C. felis* USP gene that includes the nucleic acid sequence SEQ ID NO:26, SEQ ID NO:29, SEQ ID NO:32, and/or SEQ ID NO:35 as well as the complements of any of these nucleic acid sequences. These nucleic acid sequences are further described herein. For example, nucleic acid sequence SEQ ID NO:8 represents the deduced sequence of the coding strand of a *C. felis* cDNA (complementary DNA) denoted herein as *C. felis* EcR nucleic acid molecule nECR<sub>1680</sub>, the production of which is disclosed in the Examples. Nucleic acid molecule nECR<sub>1680</sub> comprises an apparently full-length coding region. The complement of SEQ ID NO:8 (represented herein by SEQ ID NO:10) refers to the nucleic acid sequence of the strand complementary to the strand having SEQ ID NO:8, which can easily be determined by those skilled in the art. Likewise, a nucleic acid sequence complement of any nucleic acid sequence of the present invention refers to the nucleic acid sequence of the nucleic acid strand that is complementary to (i.e., can form a double helix with) the strand for which the sequence is cited. It should be noted that since nucleic acid sequencing technology is not entirely error-free, SEQ ID

NO:8 (as well as other nucleic acid and protein sequences presented herein) represents an apparent nucleic acid sequence of the nucleic acid molecule encoding an EcR protein of the present invention.

[0028] In another embodiment, an EcR gene or nucleic acid molecule can be an allelic variant that includes a similar but not identical sequence to SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, and/or SEQ ID NO:18, or any other *C. felis* EcR nucleic acid sequence cited herein and a USP gene or nucleic acid molecule can be an allelic variant that includes a similar but not identical sequence to SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, and/or SEQ ID NO:37, or any other *C. felis* USP nucleic acid sequence cited herein. For example, an allelic variant of a *C. felis* EcR gene including SEQ ID NO:8 is a gene that occurs at essentially the same locus (or loci) in the genome as the gene including SEQ ID NO:8, but which, due to natural variations caused by, for example, mutation or recombination, has a similar but not identical sequence. Because natural selection typically selects against alterations that affect function, allelic variants (i.e. alleles corresponding to, or of, cited nucleic acid sequences) usually encode proteins having similar activity to that of the protein encoded by the gene to which they are being compared. Allelic variants of genes or nucleic acid molecules can also comprise alterations in the 5' or 3' untranslated regions of the gene (e.g., in regulatory control regions), or can involve alternative splicing of a nascent transcript, thereby bringing alternative exons into juxtaposition. Allelic variants are well known to those skilled in the art and would be expected to occur naturally within a given flea such as *C. felis*, since the genome is diploid, and sexual reproduction will result in the reassortment of alleles.

[0029] In one embodiment of the present invention, isolated EcR and USP proteins are encoded by nucleic acid molecules that hybridize under stringent hybridization conditions to genes encoding flea EcR and USP proteins respectively. The minimal size of EcR and USP proteins of the present invention is a size sufficient to be encoded by a nucleic acid molecule capable of forming a stable hybrid (i.e., hybridizing under stringent hybridization conditions) with the complementary sequence of a nucleic acid molecule encoding the corresponding natural protein. The size of a nucleic acid molecule encoding such a protein is dependent on the nucleic acid composition and

the percent homology between the flea EcR or USP nucleic acid molecule and the complementary nucleic acid sequence. It can easily be understood that the extent of homology required to form a stable hybrid under stringent conditions can vary depending on whether the homologous sequences are interspersed throughout a given nucleic acid molecule or are clustered (i.e., localized) in distinct regions on a given nucleic acid molecule.

[0030] The minimal size of a nucleic acid molecule capable of forming a stable hybrid with a gene encoding a flea EcR or USP protein is typically at least about 12 to about 15 nucleotides in length if the nucleic acid molecule is GC-rich and at least about 15 to about 17 bases in length if it is AT-rich. The minimal size of a nucleic acid molecule used to encode an EcR or USP protein homolog of the present invention is from about 12 to about 18 nucleotides in length. Thus, the minimal size of EcR or USP protein homologs of the present invention is from about 4 to about 6 amino acids in length.

[0031] Stringent hybridization conditions are determined based on defined physical properties of the gene to which the nucleic acid molecule is being hybridized, and can be defined mathematically. Stringent hybridization conditions are those experimental parameters that allow an individual skilled in the art to identify significant similarities between heterologous nucleic acid molecules. These conditions are well known to those skilled in the art. See, for example, Sambrook, *et al.*

[0032] For nucleic acid molecules smaller than about 50 nucleotides, hybrid stability is defined by the dissociation temperature ( $T_d$ ), which is defined as the temperature at which 50% of the duplexes dissociate. For these smaller molecules, the stability at a standard ionic strength is defined by the following equation:  $T_d = 4(G + C) + 2(A + T)$ .

[0033] A temperature of 5 °C below  $T_d$  is used to detect hybridization between perfectly matched molecules.

[0034] Also well known to those skilled in the art is how base pair mismatch, i.e. differences between two nucleic acid molecules being compared, including non-complementarity of bases at a given location, and gaps due to insertion or deletion of

one or more bases at a given location on either of the nucleic acid molecules being compared, will affect  $T_m$  or  $T_d$  for nucleic acid molecules of different sizes. For example,  $T_m$  decreases about  $1^\circ\text{C}$  for each 1% of mismatched base pairs for hybrids greater than about 150 bp, and  $T_d$  decreases about  $5^\circ\text{C}$  for each mismatched base pair for hybrids below about 50 bp. Conditions for hybrids between about 50 and about 150 base pairs can be determined empirically and without undue experimentation using standard laboratory procedures well known to those skilled in the art. These simple procedures allow one skilled in the art to set the hybridization conditions (by altering, for example, the salt concentration, the formamide concentration or the temperature) so that only nucleic acid hybrids with greater than a specified % base pair mismatch will hybridize. Stringent hybridization conditions are commonly understood by those skilled in the art to be those experimental conditions that will allow about 30% base pair mismatch (i.e., about 70% identity). Because one skilled in the art can easily determine whether a given nucleic acid molecule to be tested is less than or greater than about 50 nucleotides, and can therefore choose the appropriate formula for determining hybridization conditions, he or she can whether the nucleic acid molecule will hybridize with a given gene under stringent hybridization conditions and similarly whether the nucleic acid molecule will hybridize under conditions designed to allow a desired amount of base pair mismatch.

[0035] Hybridization reactions are often carried out by attaching the nucleic acid molecule to be hybridized to a solid support such as a membrane, and then hybridizing with a labeled nucleic acid molecule, typically referred to as a probe, suspended in a hybridization solution. Examples of common hybridization reaction techniques include, but are not limited to, the well-known Southern and northern blotting procedures. Typically, the actual hybridization reaction is done under non-stringent conditions, i.e., at a lower temperature and/or a higher salt concentration, and then high stringency is achieved by washing the membrane in a solution with a higher temperature and/or lower salt concentration in order to achieve the desired stringency.

[0036] For example, if the skilled artisan wished to identify a nucleic acid molecule that hybridizes under stringent hybridization conditions with a *C. felis* nucleic acid molecule of about 150 bp in length, the following conditions could preferably be

used. The average G + C content of *C. felis* DNA is about 43%. The unknown nucleic acid molecules would be attached to a support membrane, and the 150 bp probe would be labeled, e.g. with a radioactive tag. The hybridization reaction could be carried out in a solution comprising 2X SSC and 0% formamide, at a temperature of about 37 ° C (low stringency conditions). Solutions of differing concentrations of SSC can be made by one of skill in the art by diluting a stock solution of 20X SSC (175.3 gram NaCl and about 88.2 gram sodium citrate in 1 liter of water, pH 7) to obtain the desired concentration of SSC. In order to achieve high stringency hybridization, the skilled artisan would calculate the washing conditions required to allow up to 30% base pair mismatch. For example, in a wash solution comprising 1X SSC and 0% formamide, the  $T_m$  of perfect hybrids would be about  $82^{\circ}\text{C} : 81.5^{\circ}\text{C} + 16.6 \log (.15\text{M}) + (0.41 \times 43) - (500/150) - (0.61 \times 0) = 82^{\circ}\text{C}$ .

[0037] Thus, to achieve hybridization with nucleic acid molecules having about 30% base pair mismatch, hybridization washes would be carried out at a temperature of about 52 ° C. It is thus within the skill of one in the art to calculate additional hybridization temperatures based on the desired percentage base pair mismatch, formulae and G/C content disclosed herein. For example, it is appreciated by one skilled in the art that as the nucleic acid molecule to be tested for hybridization against nucleic acid molecules of the present invention having sequences specified herein becomes longer than 150 nucleotides, the  $T_m$  for a hybridization reaction allowing up to 30% base pair mismatch will not vary significantly from 52 ° C.

[0038] Furthermore, it is known in the art that there are commercially available computer programs for determining the degree of similarity between two nucleic acid sequences. These computer programs include various known methods to determine the percentage identity and the number and length of gaps between hybrid nucleic acid molecules. Preferred methods to determine the percent identity among amino acid sequences and also among nucleic acid sequences include analysis using one or more of the commercially available computer programs designed to compare and analyze nucleic acid or amino acid sequences. These computer programs include, but are not limited to, GCG <sup>TM</sup> (available from Genetics Computer Group, Madison, WI), DNAsis <sup>TM</sup> (available from Hitachi Software, San Bruno, CA) and MacVector <sup>TM</sup> (available from the Eastman Kodak Company, New Haven, CT). A preferred method to determine

percent identity among amino acid sequences and also among nucleic acid sequences includes using the GAP program with pair-wise comparisons within the program GCG™ Version 9.0-UNIX, hereinafter referred to as default parameters.

[0039] Another embodiment of the present invention includes flea EcR and USP proteins. A preferred flea EcR protein includes a protein encoded by a nucleic acid molecule which is at least about 34 nucleotides and which hybridizes under conditions which preferably allow about 30% base pair mismatch, more preferably under conditions which allow about 25% base pair mismatch, more preferably under conditions which allow about 20% base pair mismatch, more preferably under conditions which allow about 15% base pair mismatch, more preferably under conditions which allow about 10% base pair mismatch and even more preferably under conditions which allow about 5% base pair mismatch with a nucleic acid molecule selected from the group consisting of SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:15, and SEQ ID NO:18.

[0040] A preferred flea USP protein includes a protein encoded by a nucleic acid molecule which is at least about 30 nucleotides and which hybridizes under conditions which preferably allow about 30% base pair mismatch, more preferably under conditions which allow about 25% base pair mismatch, more preferably under conditions which allow about 20% base pair mismatch, more preferably under conditions which allow about 15% base pair mismatch, more preferably under conditions which allow about 10% base pair mismatch and even more preferably under conditions which allow about 5% base pair mismatch with a nucleic acid molecule selected from the group consisting of SEQ ID NO:28, SEQ ID NO:31, SEQ ID NO:34, and SEQ ID NO:37.

[0041] Another embodiment of the present invention includes a flea EcR protein encoded by a nucleic acid molecule comprising at least about 34 base pairs, wherein said nucleic acid molecule hybridizes, in a solution comprising 1X SSC and 0% formamide, at a temperature of about 52 °C, to an isolated nucleic acid molecule selected from the group consisting of SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:15, and SEQ ID NO:18.

[0042] Another embodiment of the present invention includes a flea USP protein encoded by a nucleic acid molecule comprising at least about 30 base pairs, wherein said nucleic acid molecule hybridizes, in a solution comprising 1X SSC and 0% formamide, at a temperature of about 52 °C, to an isolated nucleic acid molecule selected from

the group consisting of SEQ ID NO:28, SEQ ID NO:31, SEQ ID NO:34, and SEQ ID NO:37.

[0043] Another preferred flea EcR protein of the present invention includes a protein which is encoded by a nucleic acid molecule that is preferably about 70% identical, more preferably about 75% identical, more preferably about 80% identical, more preferably about 85% identical, more preferably about 90% identical, and even more preferably about 95% identical to a nucleic acid molecule having the nucleic acid sequence SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:13, and/or SEQ ID NO:16; also preferred are fragments (i.e. portions) of such proteins encoded by nucleic acid molecules which are at least about 30 nucleotides. Percent identity as used herein is determined using the Compare function by maximum matching within the program DNAsis Version 2.1 using default parameters.

[0044] Another preferred flea USP protein of the present invention includes a protein which is encoded by a nucleic acid molecule that is preferably about 70% identical, more preferably about 75% identical, more preferably about 80% identical, more preferably about 85% identical, more preferably about 90% identical, and even more preferably about 95% identical to a nucleic acid molecule having the nucleic acid sequence SEQ ID NO:26, SEQ ID NO:29, SEQ ID NO:32, and/or SEQ ID NO:35; also preferred are fragments (i.e. portions) of such proteins encoded by nucleic acid molecules which are at least about 34 nucleotides. Percent identity as used herein is determined using the Compare function by maximum matching within the program DNAsis Version 2.1 using default parameters.

[0045] Additional preferred flea EcR proteins of the present invention include proteins having the amino acid sequence SEQ ID NO:6 or SEQ ID NO:14, and proteins comprising homologs of a protein having the amino acid sequence SEQ ID NO:6 or SEQ ID NO:14, wherein such a homolog comprises at least one epitope that elicits an immune response against a protein having an amino acid sequence SEQ ID NO:6 or SEQ ID NO:14. Likewise, also preferred are proteins encoded by nucleic acid molecules comprising nucleic acid sequence SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:13, and/or SEQ ID NO:16, or by homologs thereof.

[0046] Additional preferred flea USP proteins of the present invention include proteins

having the amino acid sequence SEQ ID NO:27 or SEQ ID NO:33, and proteins comprising homologs of a protein having the amino acid sequence SEQ ID NO:27 or SEQ ID NO:33, wherein such a homolog comprises at least one epitope that elicits an immune response against a protein having an amino acid sequence SEQ ID NO:27 or SEQ ID NO:33. Likewise, also preferred are proteins encoded by nucleic acid molecules comprising nucleic acid sequence SEQ ID NO:26, SEQ ID NO:29, SEQ ID NO:32, and/or SEQ ID NO:35, or by homologs thereof.

[0047] A preferred isolated protein of the present invention is a protein encoded by at least one of the following nucleic acid molecules: nECR<sub>2822</sub>, nECR<sub>1680</sub>, nECR<sub>666</sub>, nECR<sub>4148</sub>, nECR<sub>1683</sub>, nECR<sub>612</sub>, nUSP<sub>1749</sub>, nUSP<sub>1344</sub>, nUSP<sub>1975</sub>, nUSP<sub>1422</sub>, nUSP<sub>776</sub>, or nUSP<sub>943</sub> or allelic variants of any of these nucleic acid molecules. Another preferred isolated protein is encoded by a nucleic acid molecule having nucleic acid sequence SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:26, SEQ ID NO:29, SEQ ID NO:32, and/or SEQ ID NO:35; or a protein encoded by an allelic variant of any of these listed nucleic acid molecule.

[0048] Translation of SEQ ID NO:8, the coding strand of nECR<sub>1680</sub>, yields a protein of about 560 amino acids, denoted herein as PECD<sub>560</sub>, the amino acid sequence of which is presented in SEQ ID NO:6, assuming a first in-frame codon extending from nucleotide 1 to nucleotide 3 of SEQ ID NO:8. Sequence analysis of SEQ ID NO:6 revealed a putative DNA binding domain spanning from amino acid residue 142 to residue 207 of SEQ ID NO:6, designated SEQ ID NO:64. Sequence analysis also revealed a putative ecdysone (i.e., ligand) binding domain spanning from amino acid residue 309 to residue 527 of SEQ ID NO:6, designated SEQ ID NO:65.

[0049] Translation of SEQ ID NO:16, the coding strand of nECR<sub>1683</sub>, yields a protein of about 561 amino acids, denoted herein as PECD<sub>561</sub>, the amino acid sequence of which is presented in SEQ ID NO:14, assuming a first in-frame codon extending from nucleotide 1 to nucleotide 3 of SEQ ID NO:16. Sequence analysis of SEQ ID NO:14 revealed a putative EcR DNA binding domain spanning from amino acid residue 143 to residue 208 of SEQ ID NO:14, designated SEQ ID NO:66. Sequence analysis also revealed a putative ligand binding domain spanning from amino acid residue 310 to residue 528 of SEQ ID NO:14, designated SEQ ID NO:67.



[0050] It is within the scope of the invention that the DNA binding domains represented by SEQ ID NO:64 and SEQ ID NO:66 represent protein domains capable of binding to an ecdysone response element and the ligand binding domains represented by SEQ ID NO:65 and SEQ ID NO:67 represent protein domains capable of binding to ecdysone.

[0051] Translation of SEQ ID NO:29, the coding strand of nUSP<sub>1344</sub>, yields a protein of about 448 amino acids, denoted herein as PUSP<sub>448</sub>, the amino acid sequence of which is presented in SEQ ID NO:27, assuming a first in-frame codon extending from nucleotide 1 to nucleotide 3 of SEQ ID NO:29. Sequence analysis of SEQ ID NO:27 revealed a putative USP DNA binding domain spanning from amino acid residue 89 to residue 154 of SEQ ID NO:27, designated SEQ ID NO:68. Sequence analysis also revealed a putative ligand binding domain spanning from amino acid residue 178 to residue 448 of SEQ ID NO:27, designated SEQ ID NO:69.

[0052] Translation of SEQ ID NO:35, the coding strand of nUSP<sub>1422</sub>, yields a protein of about 474 amino acids, denoted herein as PUSP<sub>474</sub>, the amino acid sequence of which is presented in SEQ ID NO:33, assuming a first in-frame codon extending from nucleotide 1 to nucleotide 3 of SEQ ID NO:35. Sequence analysis of SEQ ID NO:33 revealed a putative DNA binding domain spanning from amino acid residue 115 to residue 180 of SEQ ID NO:33, designated SEQ ID NO:70. Sequence analysis also revealed a putative EcR (i.e., ligand) binding domain spanning from amino acid residue 204 to residue 474 of SEQ ID NO:33, designated SEQ ID NO:71.

[0053] While not being bound by theory, it is believed that the putative DNA binding domains represented by SEQ ID NO:68 and SEQ ID NO:70 and the putative ligand binding domains represented by SEQ ID NO:69 and SEQ ID NO:71 represent domains capable of affecting the binding of ecdysone receptor to ecdysone and thereby affecting DNA transcription.

[0054] Preferred proteins of the present invention include proteins that are at least about 70%, preferably at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, even more preferably at least about 95%, and even more preferably about 100% identical to PECR<sub>560</sub>, PECR<sub>561</sub>, PUSP<sub>448</sub>, or PUSP<sub>474</sub>. Additionally preferred are proteins encoded by allelic variants of a nucleic acid molecules encoding proteins PECR<sub>560</sub>, PECR<sub>561</sub>, PUSP<sub>448</sub>, or PUSP<sub>474</sub>. Also

preferred are fragments thereof having at least about 35 amino acid residues.

[0055] Other preferred EcR proteins of the present invention include proteins having amino acid sequences that are at least about 70%, preferably at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, even more preferably at least about 95%, and even more preferably about 100% identical to amino acid sequence SEQ ID NO:6 or SEQ ID NO:14. More preferred are EcR proteins comprising amino acid sequences SEQ ID NO:6 or SEQ ID NO:14; and EcR proteins encoded by allelic variants of nucleic acid molecules encoding EcR proteins having amino acid sequences SEQ ID NO:6 or SEQ ID NO:14. Also preferred are fragments thereof having at least about 35 amino acid residues.

[0056] In one embodiment of the present invention, *C. felis* EcR proteins comprise amino acid sequence SEQ ID NO:6 or SEQ ID NO:14 (including, but not limited to, the proteins consisting of amino acid sequence SEQ ID NO:6 or SEQ ID NO:14, fusion proteins and multivalent proteins), and proteins encoded by allelic variants of nucleic acid molecules encoding proteins having amino acid sequence SEQ ID NO:6 or SEQ ID NO:14. In another embodiment, *C. felis* USP proteins of the present invention comprise amino acid sequence SEQ ID NO:27 or SEQ ID NO:33 (including, but not limited to, the proteins consisting of amino acid sequence SEQ ID NO:27 or SEQ ID NO:33, fusion proteins and multivalent proteins), and proteins encoded by allelic variants of nucleic acid molecules encoding proteins having amino acid sequence SEQ ID NO:27 or SEQ ID NO:33.

[0057] In one embodiment, a preferred flea EcR protein comprises an amino acid sequence of at least about 35 amino acids, preferably at least about 50 amino acids, more preferably at least about 100 amino acids, more preferably at least about 200 amino acids, more preferably at least about 250 amino acids, more preferably at least about 300 amino acids, more preferably at least about 350 amino acids, more preferably at least about 400 amino acids, more preferably at least about 450 amino acids, more preferably at least about 500 amino acids, even more preferably at least about 550 amino acids and a preferred flea USP protein comprises an amino acid sequence of at least about 35 amino acids, preferably at least about 50 amino acids, more preferably at least about 100 amino acids, more preferably at least about 150

amino acids, more preferably at least about 200 amino acids, more preferably at least about 250 amino acids, more preferably at least about 300 amino acids, more preferably at least about 350 amino acids, more preferably at least about 400 amino acids, more preferably at least about 450 amino acids, even more preferably at least about 475 amino acids. In another embodiment, preferred fleaEcR and USP proteins comprise full-length proteins, i.e., proteins encoded by full-length coding regions.

[0058] In another embodiment, a preferred flea EcR protein comprises an isolated flea EcR protein selected from the group consisting of: (a) a protein comprising an amino acid sequence that is at least about 70% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:14, wherein said protein is at least about 71 amino acids residues in length; (b) a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:64, SEQ ID NO:65, and fragments thereof, wherein said protein has at least a portion of an EcRE binding domain; (c) a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:66, SEQ ID NO:67, and fragments thereof, wherein said protein has at least a portion of an EcR ligand binding domain or (d) a protein encoded by an allelic variant of a nucleic acid molecule which encodes any protein of (a), (b) or (c).

[0059] In another embodiment, a preferred flea USP protein comprises an isolated flea ultraspiracle protein selected from the group consisting of: (a) a protein comprising an amino acid sequence that is at least about 70% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:33, wherein said protein is at least about 72 amino acids residues in length; (b) a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, and fragments thereof, wherein said protein has at least a portion of a USP protein that is capable of affecting binding of EcR to ecdysone; or (c) a protein encoded by an allelic variant of a nucleic acid molecule which encodes any protein of (a) or (b). As used herein, the term "capable of affecting" the binding of ecdysone receptor to ecdysone means the ability of USP to act as a heterodimeric binding partner with EcR, i.e. to assist EcR in the binding of ecdysone, preferably to promote, improve and/or enhance high affinity binding between EcR and ecdysone.

[0060] One of skill in the art will understand that a DNA or protein fragment of the present invention includes a portion of a larger nucleic acid molecule or protein, respectively. Preferably, DNA fragments including the DNA binding, or ligand binding domains, of EcR can be isolated from SEQ ID NO:5 and/or SEQ ID NO:13 and DNA fragments including the DNA binding, or ligand binding domains, of USP can be isolated from SEQ ID NO:26 and/or SEQ ID NO:32. Preferably, protein fragments including the DNA binding, or ligand binding domains, of EcR can be isolated from SEQ ID NO:6 and/or SEQ ID NO:14 and protein fragments including the DNA binding, or ligand binding domains, of USP can be isolated from SEQ ID NO:27 and/or SEQ ID NO:33.

[0061] One of skill in the art will also understand that fragments including the active domains of EcR, or USP, can vary and extend beyond those particular nucleic acid or amino acid regions defined herein. Such active domains can vary in length by 1 amino acid to about 50 amino acids. Nucleic acids or amino acids essential to an active domain can be identified using standard protein or DNA binding assays known to those of skill in the art to determine the ability of an active domain to bind to its ligand, e.g. EcRE, ecdysone or EcR.

[0062] A fragment of an EcR and/or USP protein of the present invention preferably comprises at least about 5 amino acids, more preferably at least about 10 amino acids, more preferably at least about 15 amino acids, more preferably at least about 20 amino acids, more preferably at least about 25 amino acids, more preferably at least about 30 amino acids, more preferably at least about 35 amino acids, more preferably at least about 40 amino acids, more preferably at least about 45 amino acids, more preferably at least about 50 amino acids, more preferably at least about 55 amino acids, more preferably at least about 60 amino acids, more preferably at least about 65 amino acids, more preferably at least about 70 amino acids, more preferably at least about 75 amino acids, more preferably at least about 80 amino acids, more preferably at least about 85 amino acids, more preferably at least about 90 amino acids, more preferably at least about 95 amino acids, and even more preferably at least about 100 amino acids in length.

[0063] Additional preferred fragments of the present invention can include SEQ ID NO:64,

SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, and SEQ ID NO:71, as well as fragments of SEQ ID NO:6, SEQ ID NO:14, SEQ ID NO:27, and SEQ ID NO:33 that are not SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, and SEQ ID NO:71.

[0064] Additional preferred EcR and USP proteins of the present invention include proteins encoded by nucleic acid molecules comprising at least a portion of nECR<sub>2822</sub>, nECR<sub>1680</sub>, nECR<sub>4148</sub>, nECR<sub>1683</sub>, nECR<sub>612</sub>, nUSP<sub>1749</sub>, nUSP<sub>1344</sub>, nUSP<sub>1975</sub>, nUSP<sub>1422</sub>, nUSP<sub>776</sub>, and nUSP<sub>943</sub>, as well as EcR and USP proteins encoded by allelic variants of such nucleic acid molecules.

[0065] Also preferred are EcR proteins encoded by nucleic acid molecules having nucleic acid sequences comprising at least a portion of SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:13, and/or SEQ ID NO:16, as well as allelic variants of these nucleic acid molecules.

[0066] Also preferred are USP proteins encoded by nucleic acid molecules having nucleic acid sequences comprising at least a portion of SEQ ID NO:26, SEQ ID NO:29, SEQ ID NO:32, and/or SEQ ID NO:35, as well as allelic variants of these nucleic acid molecules.

[0067] In another embodiment, a preferred flea EcR protein of the present invention is encoded by a nucleic acid molecule comprising at least about 25 nucleotides, more preferably at least about 50 nucleotides, more preferably at least about 150 nucleotides, more preferably at least about 350 nucleotides, more preferably at least about 450 nucleotides, more preferably at least about 550 nucleotides, more preferably at least about 650 nucleotides, more preferably at least about 750 nucleotides, more preferably at least about 1000 nucleotides, more preferably at least about 1500 nucleotides, more preferably at least about 2000 nucleotides, more preferably at least about 2500 nucleotides, more preferably at least about 2800 nucleotides, more preferably at least about 3000 nucleotides, more preferably at least about 4000 nucleotides, and even more preferably at least about 4150 nucleotides in length, and a preferred flea USP protein of the present invention is encoded by a nucleic acid molecule comprising a coding region of at least about 25 nucleotides, more preferably at least about 50 nucleotides, more preferably at least about 100

nucleotides, more preferably at least about 150 nucleotides, more preferably at least about 250 nucleotides, more preferably at least about 500 nucleotides, more preferably at least about 800 nucleotides, more preferably at least about 1000 nucleotides, more preferably at least about 1250 nucleotides, more preferably at least about 1400 nucleotides, more preferably at least about 1750 nucleotides, more preferably at least about 1900 nucleotides, even more preferably at least about 1975 nucleotides in length. Within this embodiment is an EcR protein encoded by at least a portion of nECR<sub>2822</sub> or nECR<sub>4148</sub> or by an allelic variant of either of these nucleic acid molecules and a USP protein encoded by at least a portion of nUSP<sub>1749</sub> or nUSP<sub>1975</sub> or by an allelic variant of either of these nucleic acid molecules. In yet another embodiment, preferred flea EcR and USP proteins of the present invention are encoded by nucleic acid molecules comprising apparently full-length EcR or USP coding regions respectively, i.e., nucleic acid molecules encoding an apparently full-length EcR or USP proteins.

[0068] Preferred arthropod EcR and USP proteins of the present invention are compounds that can be used to develop inhibitors that,

[0069] Suitable fleas to target include any flea that is essentially incapable of causing disease in an animal administered an inhibitor of the present invention. As such, fleas to target includes any flea that produces a protein that can be targeted by an inhibitory compound that otherwise inhibits flea EcR or USP function (e.g., a compound that binds to flea EcR or USP thereby blocking flea development and/or migration regulatory pathways), thereby resulting in the decreased ability of the parasite to cause disease in an animal.

[0070] One embodiment of a flea EcR and/or USP protein of the present invention is a fusion protein that includes a flea EcR and/or USP protein-containing domain attached to one or more fusion segments. Suitable fusion segments for use with the present invention include, but are not limited to, segments that can: enhance a protein's stability; act as an immunopotentiator to enhance an immune response against a flea EcR and/or USP protein; and/or assist in purification of a flea EcR and/or USP protein (e.g., by affinity chromatography). A suitable fusion segment can be a domain of any size that has the desired function (e.g., imparts increased stability, imparts increased

immunogenicity to a protein, and/or simplifies purification of a protein). Fusion segments can be joined to amino and/or carboxyl termini of the flea EcR-containing and/or USP-containing domain of the protein and can be susceptible to cleavage in order to enable straight-forward recovery of a flea EcR and/or USP protein. Fusion proteins are preferably produced by culturing a recombinant cell transformed with a fusion nucleic acid molecule that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of an EcR-containing and/or USP-containing domain. Preferred fusion segments include a metal binding domain (e.g., a poly-histidine segment); an immunoglobulin binding domain (e.g., Protein A; Protein G; T cell; B cell; Fc receptor or complement protein antibody-binding domains); a sugar binding domain (e.g., a maltose binding domain); and/or a "tag" domain (e.g., at least a portion of  $\beta$ -galactosidase, a strep tag peptide, a T7 tag peptide, a Flag™ peptide, or other domains that can be purified using compounds that bind to the domain, such as monoclonal antibodies). More preferred fusion segments include metal binding domains, such as a poly-histidine segment; a maltose binding domain; a strep tag peptide, such as that available from Biometra in Tampa, FL; and an S10 peptide.

[0071] The present invention also includes mimetopes of flea EcR and/or USP proteins of the present invention. As used herein, a mimetope of a flea EcR and/or USP protein of the present invention refers to any compound that is able to mimic the activity of such an EcR and/or USP protein, often because the mimetope has a structure that mimics the particular EcR and/or USP protein. Mimetopes can be, but are not limited to: peptides that have been modified to decrease their susceptibility to degradation such as all-D retro peptides; anti-idiotypic and/or catalytic antibodies, or fragments thereof; non-proteinaceous immunogenic portions of an isolated protein (e.g., carbohydrate structures); and synthetic or natural organic molecules, including nucleic acids. Such mimetopes can be designed using computer-generated structures of proteins of the present invention. Mimetopes can also be obtained by generating random samples of molecules, such as oligonucleotides, peptides or other organic molecules, and screening such samples by affinity chromatography techniques using the corresponding binding partner.

[0072] Another embodiment of the present invention is an isolated nucleic acid molecule

comprising a flea EcR and/or USP nucleic acid molecule. The identifying characteristics of such nucleic acid molecules are heretofore described. A nucleic acid molecule of the present invention can include an isolated natural flea EcR and/or USP gene or a homolog thereof, the latter of which is described in more detail below. A nucleic acid molecule of the present invention can include one or more regulatory regions, full-length or partial coding regions, or combinations thereof. The minimal size of a nucleic acid molecule of the present invention is a size sufficient to allow the formation of a stable hybrid (i.e., hybridization under stringent hybridization conditions) with the complementary sequence of another nucleic acid molecule. As such, the minimal size of an EcR and/or USP nucleic acid molecule of the present invention is from about 12 to about 18 nucleotides in length. Suitable and preferred fleas from which to isolate nucleic acid molecules of the present invention are disclosed herein. Particularly preferred EcR and/or USP nucleic acid molecules include *C. felis*

[0073] In accordance with the present invention, an isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu (i.e., that has been subjected to human manipulation) and can include DNA, RNA, or derivatives of either DNA or RNA. As such, "isolated" does not reflect the extent to which the nucleic acid molecule has been purified. Isolated flea EcR and/or USP nucleic acid molecules of the present invention, or homologs thereof, can be isolated from a natural source or produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification or cloning) or chemical synthesis. Isolated flea EcR and/or USP nucleic acid molecules, and homologs thereof, can include, for example, natural allelic variants and nucleic acid molecules modified by nucleotide insertions, deletions, substitutions, and/or inversions in a manner such that the modifications do not substantially interfere with the nucleic acid molecule's ability to encode an EcR and/or USP protein of the present invention.

[0074] A flea EcR and/or USP nucleic acid molecule homolog can be produced using a number of methods known to those skilled in the art, see, for example, Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press; Sambrook et al., *ibid.*, is incorporated by reference herein in its entirety. For example, nucleic acid molecules can be modified using a variety of techniques including, but



not limited to, classic mutagenesis and recombinant DNA techniques such as site-directed mutagenesis, chemical treatment, restriction enzyme cleavage, ligation of nucleic acid fragments, PCR amplification, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules, and combinations thereof. Nucleic acid molecule homologs can be selected by hybridization with flea EcR or USP nucleic acid molecules or by screening the function of a protein encoded by the nucleic acid molecule (e.g., ability to elicit an immune response against at least one epitope of a flea EcR or USP protein or to effect EcR or USP activity).

[0075] An isolated nucleic acid molecule of the present invention can include a nucleic acid sequence that encodes at least one flea EcR or USP protein of the present invention, examples of such proteins being disclosed herein. Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the sequence of nucleotides on the nucleic acid molecule, the two phrases can be used interchangeably, especially with respect to a nucleic acid molecule, or a nucleic acid sequence, being capable of encoding a flea EcR or USP protein.

[0076] A preferred nucleic acid molecule of the present invention, when administered to an animal, is capable of protecting that animal from flea infestation. As will be disclosed in more detail below, such a nucleic acid molecule can be, or encode, an antisense RNA, a molecule capable of triple helix formation, a ribozyme, or other nucleic acid-based drug compound. In additional embodiments, a nucleic acid molecule of the present invention can encode a protective protein (e.g., an EcR or USP protein of the present invention), the nucleic acid molecule being delivered to the animal, for example, by direct injection (i.e., as a genetic vaccine) or in a vehicle such as a recombinant virus vaccine or a recombinant cell vaccine.

[0077] In one embodiment of the present invention, a preferred flea EcR nucleic acid molecule includes an isolated nucleic acid molecule which hybridizes under conditions which preferably allow about 30% base pair mismatch, more preferably under conditions which allow about 25% base pair mismatch, more preferably under conditions which allow about 20% base pair mismatch, more preferably under

conditions which allow about 15% base pair mismatch, more preferably under conditions which allow about 10% base pair mismatch and even more preferably under conditions which allow about 5% base pair mismatch with a nucleic acid molecule selected from the group consisting of SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:15, and SEQ ID NO:18.

[0078] In one embodiment of the present invention, a preferred flea USP nucleic acid molecule includes an isolated nucleic acid molecule which hybridizes under conditions which preferably allow about 30% base pair mismatch, more preferably under conditions which allow about 25% base pair mismatch, more preferably under conditions which allow about 20% base pair mismatch, more preferably under conditions which allow about 15% base pair mismatch, more preferably under conditions which allow about 10% base pair mismatch and even more preferably under conditions which allow about 5% base pair mismatch with a nucleic acid molecule selected from the group consisting of SEQ ID NO:28, SEQ ID NO:31, SEQ ID NO:34, and SEQ ID NO:37.

[0079] Another embodiment of the present invention includes a nucleic acid molecule, wherein said nucleic acid molecule hybridizes, in a solution comprising 1X SSC and 0% formamide, at a temperature of about 52 ° C, to an isolated nucleic acid molecule selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:37. Additional preferred nucleic acid molecules of the present invention include oligonucleotides of an isolated nucleic acid molecule, wherein said nucleic acid molecule hybridizes, in a solution comprising 1X SSC and 0% formamide, at a temperature of about 52 ° C, to an isolated nucleic acid molecule selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:37, wherein said oligonucleotide comprises at least about 30 nucleotides.

[0080] Additional preferred flea EcR nucleic acid molecules of the present invention include nucleic acid molecules comprising a nucleic acid sequence that is preferably at

least about 70%, more preferably at least about 75%, more preferably at least about 80% more preferably at least about 85%, more preferably at least about 90%, and even more preferably at least about 95% identical to a nucleic acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:16, and/or SEQ ID NO:18. Also preferred are oligonucleotides of any of such nucleic acid molecules, particularly those that are at least about 34 nucleotides. Percent identity may be determined using the program GCG Version 9.0-UNIX using default parameters.

[0081] Additional preferred flea USP nucleic acid molecules of the present invention include nucleic acid molecules comprising a nucleic acid sequence that is preferably at least about 70%, more preferably at least about 75%, more preferably at least about 80% more preferably at least about 85%, more preferably at least about 90%, and even more preferably at least about 95% identical to a nucleic acid sequence selected from the group consisting of SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, and/or SEQ ID NO:37. Also preferred are oligonucleotides of any of such nucleic acid molecules, particularly those that are at least about 30 nucleotides. Percent identity may be determined using the program GCG Version 9.0-UNIX using default parameters.

[0082] One embodiment of the present invention is a nucleic acid molecule comprising all or part of nucleic acid molecules nECR<sub>2822</sub>, nECR<sub>1680</sub>, nECR<sub>666</sub>, nECR<sub>4148</sub>, nECR<sub>1683</sub>, nECR<sub>612</sub>, nUSP<sub>1749</sub>, nUSP<sub>1344</sub>, nUSP<sub>1975</sub>, nUSP<sub>1422</sub>, nUSP<sub>776</sub>, and nUSP<sub>943</sub>, or allelic variants of these nucleic acid molecules. Another preferred nucleic acid molecule of the present invention includes at least a portion of nucleic acid sequence SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:13, SEQ ID NO:16, SEQ ID NO:26, SEQ ID NO:29, SEQ ID NO:32, and/or SEQ ID NO:35, as well as allelic variants of nucleic acid molecules having these nucleic acid sequences and homologs of nucleic acid molecules having these nucleic acid sequences; preferably such a homolog encodes or is complementary to a nucleic acid molecule that encodes at least one epitope that elicits an immune response against a protein having an amino acid sequence SEQ ID NO:6, SEQ ID NO:14, SEQ ID NO:27 or SEQ ID NO:33. Such nucleic acid molecules can include nucleotides in addition to those included in the SEQ ID NOs, such as, but not limited to, a full-length gene, a full-length coding region, a

nucleic acid molecule encoding a fusion protein, or a nucleic acid molecule encoding a multivalent protective compound.

[0083] In one embodiment, an EcR nucleic acid molecule of the present invention encodes a protein that is at least about 70%, preferably at least about 75%, more preferably at least about 80%, even more preferably at least about 85%, even more preferably at least about 90%, even more preferably at least about 95%, even more preferably at least about 98%, and even more preferably at least about 100% identical to PECR<sub>560</sub> and/or PECR<sub>561</sub>. In another embodiment, a USP nucleic acid molecule of the present invention encodes a protein that is at least about 70%, preferably at least about 75%, more preferably at least about 80%, even more preferably at least about 85%, even more preferably at least about 90%, even more preferably at least about 95%, even more preferably at least about 98%, and even more preferably at least about 100% identical to PUSP<sub>448</sub> and/or PUSP<sub>474</sub>.

[0084] In another embodiment, an EcR nucleic acid molecule of the present invention encodes a protein having an amino acid sequence that is at least about 70%, preferably at least about 75%, more preferably at least about 80%, even more preferably at least about 85%, even more preferably at least about 90%, even more preferably at least about 95%, even more preferably at least about 98%, and even more preferably at least about 100% identical to SEQ ID NO:6 or SEQ ID NO:14. The present invention also includes an EcR nucleic acid molecule encoding a protein having at least a portion of SEQ ID NO:6, and/or SEQ ID NO:14, as well as allelic variants of an EcR nucleic acid molecule encoding a protein having these sequences, including nucleic acid molecules that have been modified to accommodate codon usage properties of the cells in which such nucleic acid molecules are to be expressed.

[0085] In another embodiment, a USP nucleic acid molecule of the present invention encodes a protein having an amino acid sequence that is at least about 70%, preferably at least about 75%, more preferably at least about 80%, even more preferably at least about 85%, even more preferably at least about 90%, even more preferably at least about 95%, even more preferably at least about 98%, and even more preferably at least about 100% identical to SEQ ID NO:27 or SEQ ID NO:33. The present invention also includes a USP nucleic acid molecule encoding a protein having at least

a portion of SEQ ID NO:27, and/or SEQ ID NO:33, as well as allelic variants of a USP nucleic acid molecule encoding a protein having these sequences, including nucleic acid molecules that have been modified to accommodate codon usage properties of the cells in which such nucleic acid molecules are to be expressed.

[0086] In another embodiment, a preferred flea EcR nucleic acid molecule encodes an EcR protein comprising at least about 35 amino acids, preferably at least about 50 amino acids, more preferably at least about 100 amino acids, more preferably at least about 200 amino acids, more preferably at least about 300 amino acids, more preferably at least about 400 amino acids, more preferably at least about 500 amino acids, even more preferably at least about 560 amino acids in length.

[0087] In another embodiment, a preferred flea USP nucleic acid molecule encodes a USP protein comprising at least about at least about 35 amino acids, preferably at least about 50 amino acids, more preferably at least about 100 amino acids, more preferably at least about 200 amino acids, more preferably at least about 300 amino acids, more preferably at least about 400 amino acids, more preferably at least about 450 amino acids, even more preferably at least about 475 amino acids in length.

[0088] In another embodiment, a preferred flea EcR nucleic acid molecule comprises a nucleic acid sequence that encodes at least a portion of a flea EcR protein that is capable of binding to an ecdysone response element. Preferably, such nucleic acid molecule encodes a protein having SEQ ID NO:64 and/or SEQ ID NO:65. A preferred flea EcR protein also comprises at least a portion of a flea EcR protein that is capable of binding to ecdysone. Preferably, such nucleic acid molecule encodes a protein having SEQ ID NO:66 and/or SEQ ID NO:67.

[0089] In another embodiment, a preferred flea USP nucleic acid molecule comprises a nucleic acid sequence that encodes at least a portion of a flea USP DNA binding domain. Preferably, such nucleic acid molecule encodes a protein having SEQ ID NO:68 and/or SEQ ID NO:69. A preferred flea USP protein also comprises at least a portion of a flea USP ligand binding domain. Preferably, such nucleic acid molecule encodes a protein having SEQ ID NO:70 and/or SEQ ID NO:71.

[0090] In another embodiment, a preferred flea EcR nucleic acid molecule of the present

invention comprises an apparently full-length EcR coding region, i.e., the preferred nucleic acid molecule encodes an apparently full-length EcR protein.

[0091] In yet another embodiment, a preferred flea USP nucleic acid molecule of the present invention comprises an apparently full-length USP coding region, i.e., the preferred nucleic acid molecule encodes an apparently full-length USP protein.

[0092] Knowing the nucleic acid sequences of certain flea EcR and/or USP nucleic acid molecules of the present invention allows one skilled in the art to, for example, (a) make copies of those nucleic acid molecules, (b) obtain nucleic acid molecules including at least a portion of such nucleic acid molecules (e.g., nucleic acid molecules including full-length genes, full-length coding regions, regulatory control sequences, truncated coding regions), and (c) obtain other flea EcR and/or USP nucleic acid molecules. Such nucleic acid molecules can be obtained in a variety of ways including screening appropriate expression libraries with antibodies of the present invention; traditional cloning techniques using oligonucleotide probes of the present invention to screen appropriate libraries; and PCR amplification of appropriate libraries or DNA using oligonucleotide primers of the present invention. Preferred libraries to screen or from which to amplify nucleic acid molecules include flea 1<sup>st</sup> instar larvae; 3<sup>rd</sup> instar larvae, wandering larvae, prepupal larvae, pupae and whole adult flea cDNA libraries as well as genomic DNA libraries. Similarly, preferred DNA sources to screen or from which to amplify nucleic acid molecules include flea prepupal cDNA, adult cDNA and genomic DNA. Techniques to clone and amplify genes are disclosed, for example, in Sambrook et al., *ibid*.

[0093] The present invention also includes nucleic acid molecules that are oligonucleotides capable of hybridizing, under stringent hybridization conditions, with complementary regions of other, preferably longer, nucleic acid molecules of the present invention such as those comprising *C. felis* EcR and/or USP nucleic acid molecules or other flea EcR and/or USP nucleic acid molecules. Oligonucleotides of the present invention can be RNA, DNA, or derivatives of either. The minimum size of such oligonucleotides is the size required for formation of a stable hybrid between an oligonucleotide and a complementary sequence on a nucleic acid molecule of the present invention. A preferred oligonucleotide of the present invention has a

maximum size of preferably about 200 nucleotides, more preferably about 150 nucleotides, more preferably about 100 nucleotides and even more preferably about 50 nucleotides. The present invention includes oligonucleotides that can be used as, for example, probes to identify nucleic acid molecules, primers to produce nucleic acid molecules, or therapeutic reagents to inhibit flea EcR and/or USP protein production or activity (e.g., as antisensetriplex formationribozymeand/or RNA drug-based reagents). The present invention also includes the use of such oligonucleotides to protect animals from disease using one or more of such technologies. Appropriate oligonucleotide-containing therapeutic compositions can be administered to an animal using techniques known to those skilled in the art.

[0094] One embodiment of the present invention includes a recombinant vector, which includes at least one isolated nucleic acid molecule of the present invention, inserted into any vector capable of delivering the nucleic acid molecule into a host cell. Such a vector contains heterologous nucleic acid sequences, that is nucleic acid sequences that are not naturally found adjacent to nucleic acid molecules of the present invention and that preferably are derived from a species other than the species from which the nucleic acid molecule(s) are derived. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a virus or a plasmid. Recombinant vectors can be used in the cloning, sequencing, and/or otherwise manipulating of flea EcR and/or USP nucleic acid molecules of the present invention.

[0095] One type of recombinant vector, referred to herein as a recombinant molecule, comprises a nucleic acid molecule of the present invention operatively linked to an expression vector. The phrase operatively linked refers to insertion of a nucleic acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified nucleic acid molecule. Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can be either prokaryotic or eukaryotic, and are typically viruses or plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention, including in bacterial, fungal, parasite, insect, other animal, and plant cells. Preferred expression vectors of the present

invention can direct gene expression in bacterial, yeast, insect and mammalian cells, and more preferably in the cell types disclosed herein.

[0096] In particular, expression vectors of the present invention contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of nucleic acid molecules of the present invention. In particular, recombinant molecules of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in bacterial, yeast, or insect and mammalian cells, such as, but not limited to, *tac*, *lac*, *trp*, *trc*, oxy-pro, omp/lpp, rrnB, bacteriophage lambda (such as lambda p<sub>L</sub> and lambda p<sub>R</sub> and fusions that include such promoters), bacteriophage T7, T7 *lac*, bacteriophage T3, bacteriophage SP6, bacteriophage SP01, metallothionein, alpha-mating factor, *Pichia* alcohol oxidase, alphavirus subgenomic promoter, antibiotic resistance gene, baculovirus, *Heliothis zea* insect virus, vaccinia virus, herpesvirus, raccoon poxvirus, other poxvirus, adenovirus, cytomegalovirus (such as immediate early promoter), simian virus 40, retrovirus, actin, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences as well as other sequences capable of controlling gene expression in prokaryotic or eukaryotic cells. Additional suitable transcription control sequences include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (e.g., promoters inducible by interferons or interleukins). Transcription control sequences of the present invention can also include naturally occurring transcription control sequences naturally associated with fleas, such as *C. felis* transcription control sequences.

[0097] Suitable and preferred nucleic acid molecules to include in recombinant vectors of



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[0099] Another embodiment of the present invention includes a recombinant cell comprising a host cell transformed with one or more recombinant molecules of the present invention. Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. It is to be noted that a cell line refers to any recombinant cell of the present invention that is not a transgenic animal. Transformed nucleic acid molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained. Preferred nucleic acid molecules with which to transform a cell include *C. felis* EcR and USP nucleic acid

molecules disclosed herein. Particularly preferred nucleic acid molecules with which to transform a cell include nECR<sub>2822</sub>, nECR<sub>1680</sub>, nECR<sub>4148</sub>, nECR<sub>1683</sub>, nECR<sub>612</sub>, nUSP<sub>1749</sub>, nUSP<sub>1344</sub>, nUSP<sub>1975</sub>, nUSP<sub>1422</sub>, nUSP<sub>776</sub>, and nUSP<sub>943</sub>.

[0100] Suitable host cells to transform include any cell that can be transformed with a nucleic acid molecule of the present invention. Host cells can be either untransformed cells or cells that are already transformed with at least one nucleic acid molecule (e.g., nucleic acid molecules encoding one or more proteins of the present invention and/or other proteins useful in the production of multivalent vaccines). Host cells of the present invention either can be endogenously (i.e., naturally) capable of producing EcR and/or USP proteins of the present invention or can be capable of producing such proteins after being transformed with at least one nucleic acid molecule of the present invention. Host cells of the present invention can be any cell capable of producing at least one protein of the present invention, and include bacterial, fungal (including yeast), parasite (including helminth, protozoa and ectoparasite), other insect, other animal and plant cells. Preferred host cells include bacterial, mycobacterial, yeast, insect and mammalian cells. More preferred host cells include *Salmonella*, *Escherichia*, *Bacillus*, *Listeria*, *Saccharomyces*, *Spodoptera*, *Mycobacteria*, *Trichoplusia*, BHK (baby hamster kidney) cells, MDCK cells (Madin-Darby canine kidney cell line), CRFK cells (Crandell feline kidney cell line), CV-1 cells (African monkey kidney cell line used, for example, to culture raccoon poxvirus), COS (e.g., COS-7) cells, and Vero cells. Particularly preferred host cells are *Escherichia coli*, including *E. coli* K-12 derivatives; *Salmonella typhi*; *Salmonella typhimurium*, including attenuated strains such as UK-1  $\Pi$  3987 and SR-11  $\Pi$  4072; *Spodoptera frugiperda*; *Trichoplusia ni*; BHK cells; MDCK cells; CRFK cells; CV-1 cells; COS cells; Vero cells; and non-tumorigenic mouse myoblast G8 cells (e.g., ATCC CRL 1246). Additional appropriate mammalian cell hosts include other kidney cell lines, other fibroblast cell lines (e.g., human, murine or chicken embryo fibroblast cell lines), myeloma cell lines, Chinese hamster ovary cells, mouse NIH/3T3 cells, LMTK<sup>31</sup> cells and/or HeLa cells. In one embodiment, the proteins may be expressed as heterologous proteins in myeloma cell lines employing immunoglobulin promoters.

[0101] A recombinant cell is preferably produced by transforming a host cell with one or more recombinant molecules, each comprising one or more nucleic acid molecules of

the present invention operatively linked to an expression vector containing one or more transcription control sequences, examples of which are disclosed herein. The phrase operatively linked refers to insertion of a nucleic acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell.

[0102] A recombinant cell of the present invention includes any cell transformed with at least one of any nucleic acid molecule of the present invention. Suitable and preferred nucleic acid molecules as well as suitable and preferred recombinant molecules with which to transform cells are disclosed herein.

[0103] Recombinant cells of the present invention can also be co-transformed with one or more recombinant molecules including flea EcR and/or USP nucleic acid molecules encoding one or more proteins of the present invention and one or more other nucleic acid molecules encoding other protective compounds, as disclosed herein (e.g., to produce multivalent vaccines).

[0104] Recombinant DNA technologies can be used to improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules to high-copy number plasmids, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of nucleic acid molecules of the present invention to correspond to the codon usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporally separate recombinant cell growth from recombinant enzyme production during fermentation. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing

nucleic acid molecules encoding such a protein.

[0105] Isolated flea EcR and/or USP proteins of the present invention can be produced in a variety of ways, including production and recovery of natural proteins, production and recovery of recombinant proteins, and chemical synthesis of the proteins. In one embodiment, an isolated protein of the present invention is produced by culturing a cell capable of expressing the protein under conditions effective to produce the protein, and recovering the protein. A preferred cell to culture is a recombinant cell of the present invention. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective, medium refers to any medium in which a cell is cultured to produce a flea EcR and/or USP protein of the present invention. Such medium typically comprises an aqueous medium having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art. Examples of suitable conditions are included in the Examples section.

[0106] Depending on the vector and host system used for production, resultant proteins of the present invention may either remain within the recombinant cell; be secreted into the fermentation medium; be secreted into a space between two cellular membranes, such as the periplasmic space in *E. coli*; or be retained on the outer surface of a cell or viral membrane.

[0107] The phrase "recovering the protein", as well as similar phrases, refers to collecting the whole fermentation medium containing the protein and need not imply additional steps of separation or purification. Proteins of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential

solubilization. Proteins of the present invention are preferably retrieved in "substantially pure" form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the protein as a therapeutic composition or diagnostic. A therapeutic composition for animals, for example, should exhibit no substantial toxicity and preferably should be capable of stimulating the production of antibodies in a treated animal.

[0108] The present invention also includes isolated (i.e., removed from their natural milieu) antibodies that selectively bind to a flea EcR and/or USP protein of the present invention or a mimetope thereof (e.g., anti- *C. felis* EcR or USP antibodies). As used herein, the term "selectively binds to" an EcR and/or USP protein refers to the ability of antibodies of the present invention to preferentially bind to specified proteins and mimetopes thereof of the present invention. Binding can be measured using a variety of methods standard in the art including enzyme immunoassays (e.g., ELISA), immunoblot assays, etc.; see, for example, Sambrook et al., *ibid.*, and Harlow, et al., 1988, *Antibodies, a Laboratory Manual*, Cold Spring Harbor Labs Press; Harlow et al., *ibid.*, is incorporated by reference herein in its entirety. An anti-EcR or anti-USP antibody of the present invention preferably selectively binds to a flea EcR or USP protein respectively in such a way as to inhibit the function of that protein.

[0109] Isolated antibodies of the present invention can include antibodies in serum, or antibodies that have been purified to varying degrees. Antibodies of the present invention can be polyclonal or monoclonal, or can be functional equivalents such as antibody fragments and genetically-engineered antibodies, including single chain antibodies or chimeric antibodies that can bind to one or more epitopes.

[0110] A preferred method to produce antibodies of the present invention includes (a) administering to an animal an effective amount of a protein, peptide or mimetope thereof of the present invention to produce the antibodies and (b) recovering the antibodies. In another method, antibodies of the present invention are produced recombinantly using techniques as heretofore disclosed to produce EcR and/or USP proteins of the present invention. Antibodies raised against defined proteins or mimetopes can be advantageous because such antibodies are not substantially contaminated with antibodies against other substances that might otherwise cause

interference in a diagnostic assay or side effects if used in a therapeutic composition.

[0111] Antibodies of the present invention have a variety of potential uses that are within the scope of the present invention. For example, such antibodies can be used (a) as therapeutic compounds to passively immunize an animal in order to protect the animal from fleas susceptible to treatment by such antibodies and/or (b) as tools to screen expression libraries and/or to recover desired proteins of the present invention from a mixture of proteins and other contaminants. Furthermore, antibodies of the present invention can be used to target cytotoxic agents to fleas in order to directly kill such fleas. Targeting can be accomplished by conjugating (i.e., stably joining) such antibodies to the cytotoxic agents using techniques known to those skilled in the art. Suitable cytotoxic agents are known to those skilled in the art.

[0112] One embodiment of the present invention is a therapeutic composition that, when administered to an animal in an effective manner, is capable of protecting that animal from flea infestation. Therapeutic compositions of the present invention include at least one of the following protective compounds: an isolated antibody that selectively binds to a flea EcR or USP protein, or inhibitors of EcR and/or USP function identified by their ability to bind to a flea EcR and/or USP protein. Other protective compounds include for example, antisense-, triplex formation- ribozyme- and/or RNA drug-based technologies. As used herein, a protective compound refers to a compound that, when administered to an animal in an effective manner, is able to treat, ameliorate, and/or prevent flea infestation. Preferred fleas to target are heretofore disclosed. Examples of antibodies and inhibitors of the present invention are disclosed herein.

[0113] Additional therapeutic compositions of the present invention include a protective compound derived from a protein selected from the group consisting of: (a) an isolated flea ecdysone receptor protein selected from the group consisting of: (i) a protein comprising an amino acid sequence that is at least about 70% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:14, wherein said protein is at least about 71 amino acid residues in length; (ii) a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:64, SEQ ID NO:66, and fragments thereof, wherein said protein has at least

a portion of an ecdysone receptor DNA binding site; (iii) a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:65, SEQ ID NO:67, and fragments thereof, wherein said protein has at least a portion of an ecdysone receptor ligand binding site; and (iv) a protein encoded by an allelic variant of a nucleic acid molecule which encodes any protein of (i), (ii), or (iii); and (b) an isolated flea ultraspiracle protein selected from the group consisting of: (i) a protein comprising an amino acid sequence that is at least about 70% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:27 and SEQ ID NO:33, wherein said protein is at least about 72 amino acid residues in length; (ii) a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, and fragments thereof, wherein said protein has at least a portion of an ultraspiracle protein that is capable of affecting binding of ecdysone receptor to ecdysone; and (iii) a protein encoded by an allelic variant of a nucleic acid molecule which encodes any protein of (i) or (ii); wherein said protective compound inhibits the binding between ecdysone receptor and ecdysone. As used herein, the term "derived from" refers to a natural EcR or USP DNA or protein of the present invention, a portion of a natural EcR or USP DNA or protein of the present invention, as well as, a compound designed using an EcR or USP DNA or protein of the present invention, such as, for example, proteins encoded by recombinant DNA, peptides, antibodies or small molecule inhibitors.

[0114] Suitable inhibitors of EcR and/or USP activity are compounds that inhibit EcR and/or USP protein activity, usually by binding to or otherwise interacting with or otherwise modifying the EcR and/or USP active site. EcR and/or USP inhibitors can also interact with other regions of the EcR and/or USP protein to inhibit EcR and/or USP activity, for example, by allosteric interaction. Inhibitors of EcR and/or USP are usually relatively small compounds and as such differ from anti-EcR and anti-USP antibodies. Preferably, an EcR and/or USP inhibitor of the present invention is identified by its ability to bind to, or otherwise interact with, a flea EcR and/or USP protein, thereby inhibiting the activity of the flea EcR and/or USP.

[0115] EcR and/or USP inhibitors can be used directly as compounds in compositions of the present invention to treat animals as long as such compounds are not harmful to host animals being treated. EcR and/or USP inhibitors can also be used to identify

preferred types of flea EcR and/or USP to target using compositions of the present invention, for example by affinity chromatography. Preferred EcR and/or USP inhibitors of the present invention include, but are not limited to, flea EcR and/or USP substrate analogs, and other molecules that bind to a flea EcR and/or USP (e.g., to an allosteric site) in such a manner that EcR and/or USP activity of the flea EcR and/or USP is inhibited. An EcR and/or USP substrate analog refers to a compound that interacts with (e.g., binds to, associates with, modifies) the active site of an EcR and/or USP protein. A preferred EcR and/or USP substrate analog inhibits EcR and/or USP activity. EcR and/or USP substrate analogs can be of any inorganic or organic composition. EcR and/or USP substrate analogs can be, but need not be, structurally similar to an EcR and/or USP natural substrate as long as they can interact with the active site of that EcR and/or USP protein. EcR and/or USP substrate analogs can be designed using computer-generated structures of EcR and/or USP proteins of the present invention or computer structures of EcR's and/or USP's natural substrates. Preferred sites to model include one or more of the active sites of USP and/or EcR proteins. Substrate analogs can also be obtained by generating random samples of molecules, such as oligonucleotides, peptides, peptidomimetic compounds, or other inorganic or organic molecules, and screening such samples by affinity chromatography techniques using the corresponding binding partner, (e.g., a flea EcR and/or USP). A preferred EcR and/or USP substrate analog is a EcR and/or USP mimetic compound (i.e., a compound that is structurally and/or functionally similar to a natural substrate of an EcR and/or USP of the present invention, particularly to the region of the substrate that interacts with the EcR and/or USP active site, but that inhibits EcR and/or USP activity upon interacting with the EcR and/or USP active site).

[0116] Preferred EcR active sites include those portions of an EcR protein that binds to ecdysone, USP, and/or EcRE. Preferred USP active sites include those portions of a USP protein that binds to ecdysone, EcR, and/or EcRE.

[0117] The present invention also includes a therapeutic composition comprising at least one flea EcR and/or USP-based compound of the present invention in combination with at least one additional compound protective against one or more infectious agents. Examples of such compounds and infectious agents are disclosed herein.



[0118] In one embodiment, a therapeutic composition of the present invention can be used to protect an animal from flea infestation by administering such composition to a flea in order to prevent infestation. Such administration could be oral, or by application to the environment (e.g., spraying). Examples of such compositions include, but are not limited to, transgenic vectors capable of producing at least one therapeutic composition of the present invention. In another embodiment a flea can ingest therapeutic compositions, or products thereof, present in the blood of a host animal that has been administered a therapeutic composition of the present invention.

[0119] Therapeutic compositions of the present invention can be administered to any animal susceptible to such therapy, preferably to mammals, and more preferably to dogs, cats, humans, ferrets, horses, cattle, sheep and other pets, economic food animals, work animals and/or zoo animals. Preferred animals to protect against flea infestation include dogs, cats, humans and ferrets, with dogs and cats being particularly preferred.

[0120] In accordance with the present invention, a host animal (i.e., an animal that is or is capable of being infested with fleas) is treated by administering to the animal a therapeutic composition of the present invention in such a manner that the composition itself (e.g., an EcR and/or USP inhibitor, an EcR and/or USP synthesis suppressor (i.e., a compound that decreases the production of EcR and/or USP in fleas), an EcR and/or USP mimetope, or an anti-EcR or anti-USP antibody) or a product generated by the animal in response to administration of the composition (e.g., antibodies produced in response to administration of a flea EcR and/or USP protein or nucleic acid molecule, or conversion of an inactive inhibitor "prodrug" to an active EcR and/or USP inhibitor) ultimately enters the flea. A host animal is preferably treated in such a way that the compound or product thereof enters the blood stream of the animal. Fleas are then exposed to the composition or product when they feed from the animal. For example, flea EcR and/or USP inhibitors administered to an animal are administered in such a way that the inhibitors enter the blood stream of the animal, where they can be taken up by feeding fleas. The present invention also includes the ability to reduce larval flea infestation in that when fleas feed from a host animal that has been administered a therapeutic composition of the present invention, at least a portion of compounds of the present invention, or products thereof, in the blood

taken up by the fleas are excreted by the fleas in feces, which is subsequently ingested by flea larvae. In particular, it is of note that flea larvae obtain most, if not all, of their nutrition from flea feces. In accordance with the present invention, reducing EcR and/or USP activity in a flea can lead to a number of outcomes that reduce flea burden on treated animals and their surrounding environments. Such outcomes include, but are not limited to, (a) reducing the viability of fleas that feed from the treated animal, (b) reducing the fecundity of female fleas that feed from the treated animal, (c) reducing the reproductive capacity of male fleas that feed from the treated animal, (d) reducing the viability of eggs laid by female fleas that feed from the treated animal, (e) altering the blood feeding behavior of fleas that feed from the treated animal (e.g., fleas take up less volume per feeding or feed less frequently), (f) reducing the viability of flea larvae, for example due to the feeding of larvae from feces of fleas that feed from the treated animal and/or (g) altering the development of flea larvae (e.g., by decreasing feeding behavior, inhibiting growth, inhibiting (e.g., slowing or blocking) molting, and/or otherwise inhibiting maturation to adults).

[0121] In order to protect an animal from flea infestation, a therapeutic composition of the present invention is administered to the animal in an effective manner such that the composition is capable of protecting that animal from flea infestation. Therapeutic compositions of the present invention can be administered to animals prior to infestation in order to prevent infestation (i.e., as a preventative vaccine) and/or can be administered to animals after infestation.

[0122] Therapeutic compositions of the present invention can be formulated in an excipient that the animal to be treated can tolerate. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer, while examples of preservatives include thimerosal, - or o-cresol, formalin and benzyl alcohol. Standard formulations can either be liquid injectables or solids which

can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise dextrose, human serum albumin, preservatives, etc., to which sterile water or saline can be added prior to administration.

[0123] In one embodiment of the present invention, a therapeutic composition can include an adjuvant. Adjuvants are agents that are capable of enhancing the immune response of an animal to a specific antigen. Suitable adjuvants include, but are not limited to, cytokines, chemokines, and compounds that induce the production of cytokines and chemokines (e.g., granulocyte macrophage colony stimulating factor (GM-CSF), Flt-3 ligand, granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), colony stimulating factor (CSF), erythropoietin (EPO), interleukin 2 (IL-2), interleukin-3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), interferon gamma, interferon gamma inducing factor I (IGIF), transforming growth factor beta, RANTES (regulated upon activation, normal T cell expressed and presumably secreted), macrophage inflammatory proteins (e.g., MIP-1 alpha and MIP-1 beta), and Leishmania elongation initiating factor (LEIF)); bacterial components (e.g., endotoxins, in particular superantigens, exotoxins and cell wall components); aluminum-based salts; calcium-based salts; silica; polynucleotides; toxoids; serum proteins, viral coat proteins; block copolymer adjuvants (e.g., Hunter's Titermax™ adjuvant (Vaxcel™, Inc. Norcross, GA), Ribi adjuvants (Ribi ImmunoChem Research, Inc., Hamilton, MT); and saponins and their derivatives (e.g., Quil A (Superfos Biosector A/S, Denmark). Protein adjuvants of the present invention can be delivered in the form of the protein themselves or of nucleic acid molecules encoding such proteins using the methods described herein.

[0124] In one embodiment of the present invention, a therapeutic composition can include a carrier. Carriers include compounds that increase the half-life of a therapeutic composition in the treated animal. Suitable carriers include, but are not limited to, polymeric controlled release vehicles, biodegradable implants, liposomes, bacteria, viruses, other cells, oils, esters, and glycols.

[0125] One embodiment of the present invention is a controlled release formulation that

is capable of slowly releasing a composition of the present invention into an animal. As used herein, a controlled release formulation comprises a composition of the present invention in a controlled release vehicle. Suitable controlled release vehicles include, but are not limited to, biocompatible polymers, other polymeric matrices, capsules, microcapsules, microparticles, bolus preparations, osmotic pumps, diffusion devices, liposomes, lipospheres, and transdermal delivery systems. Other controlled release formulations of the present invention include liquids that, upon administration to an animal, form a solid or a gel *in situ*

[0126] A preferred controlled release formulation of the present invention is capable of releasing a composition of the present invention into the blood of the treated animal at a constant rate sufficient to attain therapeutic dose levels of the composition to protect an animal from flea infestation. The therapeutic composition is preferably released over a period of time ranging from about 1 to about 12 months. A controlled release formulation of the present invention is capable of effecting a treatment preferably for at least about 1 month, more preferably for at least about 3 months, even more preferably for at least about 6 months, even more preferably for at least about 9 months, and even more preferably for at least about 12 months.

[0127] Therapeutic compositions of the present invention can be administered to animals prior to infestation in order to prevent infestation and/or can be administered to animals after infestation. For example, proteins, mimetopes thereof, and antibodies thereof can be used as immunotherapeutic agents. Acceptable protocols to administer therapeutic compositions in an effective manner include individual dose size, number of doses, frequency of dose administration, and mode of administration. Determination of such protocols can be accomplished by those skilled in the art. A suitable single dose is a dose that is capable of protecting an animal from disease when administered one or more times over a suitable time period. For example, a preferred single dose of a protein, mimetope or antibody therapeutic composition is from about 1 microgram ( $\mu$ g) to about 10 milligrams (mg) of the therapeutic composition per kilogram body weight of the animal. Booster vaccinations can be administered from about 2 weeks to several years after the original administration. Booster administrations preferably are administered when the immune response of animal becomes insufficient to protect the animal from disease. A preferred

administration schedule is one in which from about 10  $\mu$ g to about 1 mg of the therapeutic composition per kg body weight of the animal is administered from about one to about two times over a time period of from about 2 weeks to about 12 months. Modes of administration can include, but are not limited to, subcutaneous, intradermal, intravenous, intranasal, oral, transdermal and intramuscular routes.

[0128] According to one embodiment, a nucleic acid molecule of the present invention can be administered to an animal in a fashion to enable expression of that nucleic acid molecule into a protective protein or protective RNA (e.g., antisense RNA, ribozyme, triple helix forms or RNA drug) in the animal. Nucleic acid molecules can be delivered to an animal in a variety of methods including, but not limited to, (a) administering a naked (i.e., not packaged in a viral coat or cellular membrane) nucleic acid as a genetic vaccine (e.g., as naked DNA or RNA molecules, such as is taught, for example in Wolff et al., 1990, *Science* 247, 1465-1468) or (b) administering a nucleic acid molecule packaged as a recombinant virus vaccine or as a recombinant cell vaccine (i.e., the nucleic acid molecule is delivered by a viral or cellular vehicle).

[0129] A genetic (i.e., naked nucleic acid) vaccine of the present invention includes a nucleic acid molecule of the present invention and preferably includes a recombinant molecule of the present invention that preferably is replication, or otherwise amplification, competent. A genetic vaccine of the present invention can comprise one or more nucleic acid molecules of the present invention in the form of, for example, a dicistronic recombinant molecule. Preferred genetic vaccines include at least a portion of a viral genome (i.e., a viral vector). Preferred viral vectors include those based on alphaviruses, poxviruses, adenoviruses, herpesviruses, picornaviruses, and retroviruses, with those based on alphaviruses (such as sindbis or Semliki forest virus), species-specific herpesviruses and poxviruses being particularly preferred. Any suitable transcription control sequence can be used, including those disclosed as suitable for protein production. Particularly preferred transcription control sequences include cytomegalovirus immediate early (preferably in conjunction with Intron-A), Rous sarcoma virus long terminal repeat, and tissue-specific transcription control sequences, as well as transcription control sequences endogenous to viral vectors if viral vectors are used. The incorporation of a "strong" polyadenylation signal is also preferred.

[0132] When administered to an animal, a recombinant virus vaccine of the present invention infects cells within the immunized animal and directs the production of a protective protein or RNA nucleic acid molecule that is capable of protecting the animal from flea infestation as disclosed herein. For example, a recombinant virus vaccine comprising an EcR and/or USP nucleic acid molecule of the present invention is administered according to a protocol that results in the animal producing a sufficient immune response to protect itself from flea infestation. A preferred single dose of a recombinant virus vaccine of the present invention is from about  $1 \times 10^4$  to about  $1 \times 10^8$  virus plaque forming units (pfu) per kilogram body weight of the animal. Administration protocols are similar to those described herein for protein-based vaccines, with subcutaneous, intramuscular, intranasal and oral administration routes

being preferred.

[0133] A recombinant cell vaccine of the present invention includes recombinant cells of the present invention that express at least one protein of the present invention. Preferred recombinant cells for this embodiment include *Salmonella*, *E. coli*, *Listeria*, *Mycobacterium*, *S. frugiperda*, yeast, (including *Saccharomyces cerevisiae* and *Pichia pastoris*), BHK, CV-1, myoblast G8, COS (e.g., COS-7), Vero, MDCK and CRFK recombinant cells. Recombinant cell vaccines of the present invention can be administered in a variety of ways but have the advantage that they can be administered orally, preferably at doses ranging from about  $10^8$  to about  $10^{12}$  cells per kilogram body weight. Administration protocols are similar to those described herein for protein-based vaccines. Recombinant cell vaccines can comprise whole cells, cells stripped of cell walls or cell lysates.

[0134] The efficacy of a therapeutic composition of the present invention to protect an animal from flea infestation can be tested in a variety of ways including, but not limited to, detection of protective antibodies (using, for example, proteins or mimetopes of the present invention), detection of cellular immunity within the treated animal, or challenge of the treated animal with the fleas to determine whether the treated animal is resistant to infestation. Challenge studies can include direct administration of fleas to the treated animal. In one embodiment, therapeutic compositions can be tested in animal models such as mice. Such techniques are known to those skilled in the art.

[0135] One therapeutic composition of the present invention includes an inhibitor of flea EcR and/or USP activity, i.e., a compound capable of substantially interfering with the function of a flea EcR and/or USP susceptible to inhibition by an inhibitor of flea EcR and/or USP activity. An inhibitor of EcR and/or USP activity can be identified using flea EcR and/or USP proteins of the present invention. One embodiment of the present invention is a method to identify a compound capable of inhibiting EcR and/or USP activity of a flea. Such a method includes the steps of (a) contacting (e.g., combining, mixing) an isolated flea EcR and/or USP protein, preferably a *C. felis* EcR and/or USP protein of the present invention, with a putative inhibitory compound under conditions in which, in the absence of the compound, the protein has EcR and/or USP

activity, and (b) determining if the putative inhibitory compound inhibits the EcR and/or USP activity. As used herein, the term "EcR activity" means the ability of EcR to bind to or otherwise interact with ecdysone, USP and/or EcRE and thereby affect DNA transcription. As used herein, the term "USP activity" means the ability of USP to bind to or otherwise interact with ecdysone, EcR and/or EcRE, preferably the ability to affect the association of EcR with ecdysone, more preferably the ability to promote, improve and/or enhance the association between EcR and ecdysone, thereby affecting DNA transcription.

[0136] Another embodiment of a method to identify a compound capable of inhibiting EcR and/or USP activity of a flea includes the steps of (a) contacting an isolated flea EcR and/or USP protein, preferably a *C. felis* EcR and/or USP protein of the present invention, with a putative inhibitory compound under conditions in which the EcR and/or USP protein can bind to the putative inhibitory compound, and (b) determining if the putative inhibitory compound binds to the EcR and/or USP protein.

[0137] Putative inhibitory compounds to screen include small organic molecules, antibodies (including mimetopes thereof) and substrate analogs. Methods to determine EcR and/or USP activity are known to those skilled in the art; see, for example, the Examples section of the present application. Methods to determine binding of a putative inhibitory compounds to an EcR and/or USP protein are known to those of skill in the art and include, for example, determining changes in molecular mass using surface plasmon resonance (e.g., determining light scatter by an inhibitor or an EcR and/or USP protein, before and after contacting the inhibitor or protein with an EcR and/or USP protein or inhibitor, respectively).

[0138] One embodiment of the present invention is a method to identify proteins that specifically interact with an EcR or USP protein of the present invention. The method can comprise the steps of a) identifying and isolating a protein-binding domain of an isolated flea EcR or USP protein; b) contacting that protein-binding domain with isolated flea proteins under conditions such that a flea protein and the protein-binding domain can selectively interact and/or bind to each other, using, for example, the yeast two-hybrid system see, for example, Luban, et al., 1995, *Curr. Opin. Biotechnol.*, 6, 59-64; and c) identifying those proteins that specifically bind to the



isolated EcR or USP protein-binding domain. Additional methods to identify protein-protein interactions with the protein-binding domains of an isolated EcR or USP protein of the present invention are known to those skilled in the art. Examples include Biacore ® screening, confocal immunofluorescent microscopy, and immunoprecipitations.

[0139] An inhibitor of EcR and/or USP function can be identified using flea EcR and/or USP proteins of the present invention. A preferred inhibitor of EcR and/or USP function is a compound capable of substantially interfering with the function of a flea EcR and/or USP protein and which does not substantially interfere with host animal EcR and/or USP activity. As used herein, a compound that does not substantially inhibit host animal EcR and/or USP activity is one that, when administered to a host animal, the host animal shows no significant adverse effects attributable to the compound and which, when administered to an animal in an effective manner, is capable of protecting that animal from flea infestation.

[0140] A preferred method to identify a compound capable of inhibiting EcR and/or USP activity includes contacting an isolated flea EcR and/or USP protein having an amino acid sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:14, SEQ ID NO:27, and SEQ ID NO:33 with a putative inhibitory compound under conditions in which, in the absence of said compound, said protein has EcR and/or USP activity; and determining if said putative inhibitory compound inhibits said activity. An additional preferred method of identifying a compound capable of inhibiting flea EcR and/or USP activity includes contacting an isolated host animal EcR and/or USP protein with the putative EcR and/or USP inhibitory compound under conditions in which, in the absence of said compound, said host animal EcR and/or USP protein has EcR and/or USP activity; and determining if said putative inhibitory compound inhibits the host animal EcR and/or USP activity.

[0141] The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention. The following examples include a number of recombinant DNA and protein chemistry techniques known to those skilled in the art; see, for example, Sambrook et al., *ibid*.

## Example 1

[0142] This Example describes the preparation of a head and nerve cord cDNA pool from the flea *Ctenocephalides felis*.

[0143] A flea head and nerve cord cDNA pool was prepared using Clontech's MARATHON™ cDNA Amplification kit and protocol, available from Clontech Laboratories, Palo Alto, CA. Briefly, head and nerve cords from 100 fed and 100 unfed adult fleas were isolated and about 8 µg of total RNA was extracted and used for a first strand cDNA synthesis reaction with AMV reverse transcriptase. Five microliters (µl) of the first reaction product was used as the template in a second strand cDNA reaction, using Clontech's second strand enzyme cocktail and protocols, to yield double stranded cDNA. Marathon cDNA adaptors were ligated to double stranded cDNA using T4 DNA ligase according to the manufacturer's instructions.

## Example 2

[0144] This example describes the cloning and sequencing of flea ecdysone receptor (EcR) nucleic acid molecules.

[0145]

Degenerate primers were designed based on several conserved regions of published EcR amino acid sequences of *Bombyx mori*, Swevers et al., 1995, *ibid.*, *Drosophila melanogaster*, Koelle et al., 1991, *ibid.*, and *Manduca sexta*, Fujiwara et al., 1995, *ibid.*, and human retinoic acid receptor alpha-1 sequence, Giguere et al., 1987, *ibid.* Sense primer JER-2, having the nucleotide sequence 5' TGY GAA ATG GAY ATG TAY ATG 3' (wherein Y represents C or T), designated herein as SEQ ID NO:44, was used in combination with antisense primer JER-4, having the nucleotide sequence 5' CCY TTW GCR AAT TCN ACD AT 3' (wherein Y represents C or T, W represents A or T, R represents A or G, N represents A, T, C or G, and D represents A or G or T), designated herein as SEQ ID NO:45, to produce a PCR product from a flea mixed instar cDNA library, prepared as described in Example 11 of PCT Publication WO 98/21324. PCR reaction were performed using the following amplification cycles: (1) one cycle at 95 ° C for three minutes; (2) thirty-five cycles at 95 ° C for thirty seconds, 50 ° C for thirty seconds, and 72 ° C for one minute; and (3) one cycle of 72 ° C for nine minutes, in reactions containing 1.5 millimolar (mM) MgCL<sub>2</sub>, 0.2 mM dNTPs, 1 µM of each primer, 1 µl of 5 units per microliter (U/ µl) *Taq* polymerase, and 1 µl of

template. The reaction product was re-amplified under the same reaction conditions except that part (2) ran for only twenty-five cycles. The resulting PCR amplification product was a fragment of about 446 nucleotides, denoted herein as nECR<sub>446</sub>. The PCR product was purified using Qiagen's Qiaquick™ kit using the manufacturer's protocol, available from Qiagen, Chatsworth, CA, and sequenced using primers JER-2 and JER-4 using standard sequencing methods. The resulting nucleic acid sequence of nECR<sub>446</sub> has a coding strand presented herein as SEQ ID NO:1 and a complementary strand presented herein as SEQ ID NO:2.

[0146] nECR<sub>446</sub> was used as the template for a second PCR reaction using sense primer BER-1, having nucleotide sequence 5' GGT TCC CGA AAA CCA ATG 3', designated herein as SEQ ID NO:46, and anti-sense primer BER-2, having nucleotide sequence 5' GCC GAA ATT CAA GAG CTT C 3', designated herein as SEQ ID NO:47. PCR reactions were performed using the following amplification cycles: (1) one cycle at 95 ° C for two minutes and forty seconds; (2) thirty-five cycles at 95 ° C for thirty seconds, 52.8 ° C for thirty seconds, and 72 ° C for one minute; and (3) one cycle at 72 ° C for eight minutes, in reactions containing 1.5 mM MgCL<sub>2</sub>, 0.2 mM dNTPs, 1 μ M of each primer, 1 μ l of 5U/ μ l *Taq* polymerase, and 1 Φ l of template. The resulting PCR amplification product was a fragment of about 350 nucleotides, denoted herein as nECR<sub>350</sub>. The PCR product was purified using the Qiaquick™ kit and sequenced using primers BER-1 and BER-2 using standard sequencing methods. The resulting nucleic acid sequence of nECR<sub>350</sub> has a coding strand presented herein as SEQ ID NO:3 and a complementary strand presented herein as SEQ ID NO:4.

[0147] A DNA probe comprising nucleotides from nECR<sub>350</sub>, SEQ ID NO:3, was labeled with <sup>32</sup>P and used to screen about 300,000 plaques from the flea mixed instar cDNA library and a flea pre-pupal cDNA library prepared as described in Example 11 of PCT Publication WO 98/21324. The following hybridization conditions were used. Filters were hybridized with about 1 X 10<sup>6</sup> counts per minute (cpm) per ml of the probe in 5X SSPE, 1% Sarcosyl, 0.1 mg/ml salmon sperm DNA and 0.1 mg/ml BLOTTO at 45 ° C for about 14 hours. The filters were washed twice for 30 minutes per wash in 500 ml of 5X SSPE, 1% Sarcosyl at 45 ° C, hereinafter referred to as "standard Ecr hybridization conditions". A positive plaque, denoted herein as EcR3 was further screened to obtain a pure plaque population. *In vivo* excision was performed using the

Stratagene Ex-Assist™ helper phage system and protocols, to convert ten positive plaques to pBluescript plasmid DNA. Multiple clones were sequenced following preparation with a Qiagen Qiaprep™ spin mini prep kit using the manufacturer's instructions and restriction enzyme digestion with about 20 U/ μl each of *Eco* RI and *Xho* I, available from New England Biolabs, Beverly, MA. A clone was isolated from a tertiary plaque of EcR3, containing a nucleic acid molecule of about 2822 base pairs, referred to herein as nECR<sub>2822</sub>, having a nucleotide sequence denoted herein as SEQ ID NO:5. The complement of SEQ ID NO:5 is represented herein as SEQ ID NO:7.

[0148] Translation of SEQ ID NO:5 suggests that nucleic acid molecule nECR<sub>2822</sub> encodes a full-length EcR protein of 560 amino acids, referred to herein as PECR<sub>560</sub>, having an amino acid sequence represented by SEQ ID NO:6, assuming the initiation codon spans from nucleotide 605 through nucleotide 607 of SEQ ID NO:5 and the termination codon spans from nucleotide 2285 through nucleotide 2287 of SEQ ID NO:5. The coding region encoding PECR<sub>560</sub>, is represented by nucleic acid molecule nECR<sub>1680</sub>, having a coding strand with the nucleic acid sequence represented by SEQ ID NO:8 and a complementary strand with nucleic acid sequence represented by SEQ ID NO:10. The amino acid sequence of PECR<sub>560</sub> predicts that PECR<sub>560</sub> has an estimated molecular weight of about 61.8 kilodaltons (kDa) and an estimated pI of about 6.5. A DNA probe comprising nucleotide 318 through nucleotide 2287 of SEQ ID NO:5 was labeled with <sup>32</sup>P and used to probe separate samples of *C. felis* genomic DNA which had been digested with *Eco* RI and *Eco* RV, respectively. One to three bands of digested DNA hybridized with labeled probes, under standard EcR hybridization conditions described herein indicating that each of these genes are single copy number in genes.

[0149] Comparison of amino acid sequence SEQ ID NO:6 with amino acid sequences reported in GenBank indicates that SEQ ID NO:6 showed the most homology, i.e., about 64% identity between SEQ ID NO:6 and a *Drosophila melanogaster* EcR protein isoform B1, GenBank Accession No. P34021. Comparison of SEQ ID NO:8 with nucleic acid sequences reported in GenBank indicates that SEQ ID NO:8 showed the most homology, i.e., about 63% identity between SEQ ID NO:8 and a *Lucilia cuprina* EcR nucleic acid molecule, GenBank Accession number U75377. Percent identity calculations were performed using GCG version 9.0-UNIX using default parameters.

[0150] An isoform of flea EcR was isolated as follows. Primer BER-10, having the nucleotide sequence 5' GTC AGG AAT GTA GGC TCA 3', designated herein as SEQ ID NO:48 and corresponding to nucleotides 1015 through 1032 of nucleic acid molecule nECR<sub>2822</sub> was used in combination with vector primer T3, having the nucleotide sequence 5' AAT TAA CCC TCA CTA AAG GG 3', designated herein as SEQ ID NO:49, to generate a PCR product from a primary phage plaque, denoted EcR8, which hybridized to nECR<sub>350</sub> using standard EcR hybridization conditions. PCR reaction were performed using the following amplification cycles: (1) one cycle at 95 ° C for two minutes and forty seconds; (2) thirty-five cycles at 95 ° C for thirty seconds, 50 ° C for one minute, and 72 ° C for two minutes; and (3) one cycle at 72 ° C for eight minutes, in reactions containing 1.5 mM MgCL<sub>2</sub>, 0.2 mM dNTPs, 1 µ M of each primer, 1 µ l of 5U/ µ l *Taq* polymerase, and 1 µ l of template, hereinafter referred to as "standard PCR conditions". The resulting PCR amplification product was a fragment of about 666 base pairs, denoted herein as nECR<sub>666</sub>. The PCR product was purified using the Qiaquick™ kit and sequenced using primers BER-10 and T3 using standard sequencing methods. The resulting nucleic acid sequence of nECR<sub>666</sub> has a coding strand presented herein as SEQ ID NO:11 and a complementary strand presented herein as SEQ ID NO:12.

[0151] A DNA probe comprising nucleotides from nECR<sub>666</sub>, SEQ ID NO:11, was labeled with <sup>32</sup>P, and used to re-screen EcR8 primary phage plaques until a pure plaque population was obtained. *In vivo* excision was performed using Stratagene Ex-Assist™ helper phage system and protocols, to convert positive plaques to pBluescript plasmid DNA. Multiple clones were sequenced following preparation with the Qiaprep™ spin mini prep kit and restriction enzyme digestion with 20 U/ µ l each of *Eco* RI and *Xho* I. A clone was isolated having an about 4148 base pair insert, referred to herein as nECR<sub>4148</sub>, having a nucleotide sequence denoted herein as SEQ ID NO:13. The complement of SEQ ID NO:13 is represented herein by SEQ ID NO:15.

[0152] Translation of SEQ ID NO:13 suggests that nucleic acid molecule nECR<sub>4148</sub> encodes a full-length EcR protein of 561 amino acids, referred to herein as PEcR<sub>561</sub>, having an amino acid sequence represented by SEQ ID NO:14, assuming the initiation codon spans from nucleotide 184 through nucleotide 186 of SEQ ID NO:13 and the termination codon spans from nucleotide 1867 through nucleotide 1869 of SEQ ID

NO:13. The coding region encoding PE<sub>CR</sub><sub>561</sub>, is represented by nucleic acid molecule nE<sub>CR</sub><sub>1683</sub>, having a coding strand with the nucleic acid sequence represented by SEQ ID NO:16 and a complementary strand with nucleic acid sequence represented by SEQ ID NO:18. The amino acid sequence of PE<sub>CR</sub><sub>561</sub> predicts that PE<sub>CR</sub><sub>561</sub> has an estimated molecular weight of about 62.6 kDa and an estimated pI of about 7.

[0153] Comparison of amino acid sequence SEQ ID NO:14 with amino acid sequences reported in GenBank indicates that SEQ ID NO:14 showed the most homology, i.e., about 66% identity between SEQ ID NO:14 and a *Drosophila melanogaster* EcR protein isoform A, GenBank Accession No. P34021. Comparison of SEQ ID NO:16 with nucleic acid sequences reported in GenBank indicates that SEQ ID NO:16 showed the most homology, i.e., about 59% identity between SEQ ID NO:16 and a *Lucilia cuprina* EcR nucleic acid molecule, GenBank Accession No. U75355. A comparison of nE<sub>CR</sub><sub>2822</sub> and nE<sub>CR</sub><sub>4148</sub> indicates that these molecules represent different variants of EcR in *C. felis*. Percent identity calculations were performed using GCG version 9.0-UNIX using default parameters.

### Example 3

[0154] This example describes the cloning and sequencing of flea ultraspiracle (USP) nucleic acid molecules.

[0155] Degenerate primers were designed based on several conserved regions of published USP amino acid sequences of *Bombyx mori*, Tzertzinis et al., 1994, *ibid.*, *Drosophila melanogaster*, Oro et al., 1990, *ibid.*, and *Manduca sexta*, Jindra et al., *ibid.*, published amino acid sequences of human retinoic acid receptor RXR- $\gamma$ , Cooke et al., 1996, *ibid.*, mouse retinoic acid receptor RXR- $\gamma$ , Leid et al., 1992, *ibid.*, and *Xenopus laevis* retinoic acid receptor RXR- $\alpha$ , Blumberg et al., 1992, *ibid.* Sense primer B-USP-1 having the nucleotide sequence 5' GGW AAA CAY TAT GGW GTW TA 3' (wherein W represents A or T, and Y represents C or T), designated herein as SEQ ID NO:50, was used in combination with antisense primer B-USP-3, having the nucleotide sequence 5' TTC TTC YTG NAC WHC TTC 3' (wherein Y represents C or T, N represent A or T or C or G, and W represents A or T), designated herein as SEQ ID NO:51, to produce a PCR product from the flea pre-pupal cDNA

library, using standard PCR conditions described in Example 2. The resulting PCR amplification product was a fragment of about 160 nucleotides, denoted herein as nUSP<sub>160</sub>. The PCR product was purified using Qiagen's Qiaquick™ kit and protocol and cloned into the pCRII TA™ vector, available from Invitrogen, San Diego, CA, according to the manufacturer's instructions. Clones were prepared using Qiagen's QIAprep™ spin mini prep kit and protocol and screened by restriction enzyme digest using 20 U/ μl *Eco* RI. One screened clone was isolated and sequenced using TA+ and TA- primers, available from Invitrogen. The resulting nucleic acid sequence of nUSP<sub>160</sub> has a coding strand presented herein as SEQ ID NO:19 and a complementary strand presented herein as SEQ ID NO:20.

[0156] A DNA probe comprising nucleotides from nUSP<sub>160</sub>, SEQ ID NO:19, was labeled with <sup>32</sup>P and used to screen about 450,000 plaques from the flea pre-pupal cDNA library described in Example 2, using the following hybridization conditions. Filters were hybridized with about 1 X 10<sup>6</sup> counts per minute (cpm) per ml of the probe in 5X SSPE, 1% Sarcosyl, 0.1 mg/ml salmon sperm DNA and 0.1 mg/ml BLOTTO at 45 ° C for about 14 hours. The filters were washed twice for 30 minutes per wash in 500 ml of 5X SSPE, 1% Sarcosyl at 45 ° C, hereinafter referred to as "standard USP hybridization conditions". Two positive plaques, denoted herein as USP11 and USP12, were further screened to obtain pure plaque populations of each plaque. *In vivo* excision was performed using Stratagene Ex-Assist™ helper phage system and protocols, to convert positive plaques to pBluescript plasmid DNA. Clones USP11 and USP12 were sequenced following preparation with the Qiaprep™ spin mini prep kit and restriction enzyme digestion with 20 U/ μl each of *Eco* RI and *Xho* I. A clone from plaque USP11 was isolated having an about 1421 base pair insert, referred to herein as nUSP<sub>1421</sub>, having a nucleotide sequence denoted herein as SEQ ID NO:23. The complement of SEQ ID NO:23 is represented herein by SEQ ID NO:24.

[0157] Sequence analysis revealed that nUSP<sub>1421</sub> was truncated at the 5' end. Additional 5' sequence was determined as follows. Antisense primer B-USP-5, having nucleotide sequence 5' TTC TCG TTT CAT TCC ACA GG 3', designated herein as SEQ ID NO:52, which corresponds to nucleotides 141 to 160 of nUSP<sub>160</sub>, was used in combination with primer T3, SEQ ID NO:49, to create a PCR product using the primary USP11 phage plug as the template and standard PCR conditions. The resulting about 819 base pair

PCR product, referred to herein as nUSP<sub>819</sub>, designated herein as SEQ ID NO:25, was sequenced and nucleotides 646 through 819 of nUSP<sub>819</sub> were found to overlap with nucleotides 11 through 185 of nUSP<sub>1421</sub>.

[0158] Primers based upon the combined sequences of nUSP<sub>1421</sub> and nUSP<sub>819</sub>, were used to produce a PCR product from the flea pre-pupal cDNA library containing a non-truncated 5' end. Sense primer USP11-5O, having nucleotide sequence 5' AAA GGG AAC AAA AGC TGG AGC TCC ACC GC 3', designated herein as SEQ ID NO:53, was used in combination with antisense primer USP11-3O, having the nucleotide sequence 5' TTA AAA TAT CAC TGG TTC GTA TCC TCC C 3', designated herein as SEQ ID NO:54, to produce the PCR product. The product from this first PCR reaction was used as the template in a second PCR reaction using sense primer USP11-5I, having the nucleotide sequence 5' GGC GGC CGC TCT AGA ACT AGT GGA TC 3', designated herein as SEQ ID NO:55, and antisense primer USP11-3I, having the nucleotide sequence 5' AGA CAA TCA ATA TCC CAA GTG CG 3', designated herein as SEQ ID NO:56, under standard PCR conditions as described in Example 2. The resulting PCR product was a fragment of about 1749base pairs, denoted herein as nUSP<sub>1749</sub>. The PCR product was purified using the Qiaquick™ kit and cloned into the pCRII TA™ vector, using the manufacturer's instructions. Clones were prepared using the QIAprep™ spin mini prep kit and preferred clones were identified by restriction enzyme digestion using 20 U/ μl *Eco* RI. One clone was isolated and sequenced using TA+ and TA- primers. The resulting nucleic acid sequence of nUSP<sub>1749</sub> has a coding strand presented herein as SEQ ID NO:26 and a complementary strand presented herein as SEQ ID NO:28.

[0159] Translation of SEQ ID NO:26 suggests that nucleic acid molecule nUSP<sub>1749</sub> encodes a full-length USP protein of 448 amino acids, referred to herein as PUSP<sub>448</sub>, having an amino acid sequence represented by SEQ ID NO:27, assuming the initiation codon spans from nucleotide 306 through nucleotide 308 of SEQ ID NO:26 and the termination codon spans from nucleotide 1650 through nucleotide 1652 of SEQ ID NO:26. The coding region encoding PUSP<sub>448</sub>, is represented by nucleic acid molecule nUSP<sub>1344</sub>, having a coding strand with the nucleic acid sequence represented by SEQ ID NO:29 and a complementary strand with nucleic acid sequence represented by SEQ ID NO:31. The amino acid sequence of PUSP<sub>448</sub> predicts that PUSP<sub>448</sub> has an estimated molecular weight of about 49.6 kDa and an estimated pI of



about 8.

[0160] Comparison of amino acid sequence SEQ ID NO:27 with amino acid sequences reported in GenBank indicates that SEQ ID NO:27 showed the most homology, i.e., about 58% identity between SEQ ID NO:27 and a *Drosophila melanogaster* steroid hormone receptor like protein, GenBank Accession No. S13119. Comparison of SEQ ID NO:29 with nucleic acid sequences reported in GenBank indicates that SEQ ID NO:29 showed the most homology, i.e., about 57% identity between SEQ ID NO:29 and a *Manduca sexta* USP-1 nucleic acid molecule, GenBank Accession No. U44837. Percent identity calculations were performed using GCG version 9.0-UNIX using default parameters.

[0161] A clone from plaque USP12 was isolated having an about 2149 base pair insert, referred to herein as nUSP<sub>2149</sub>, having a nucleotide sequence denoted herein as SEQ ID NO:21. The complement of SEQ ID NO:21 is represented herein by SEQ ID NO:22. Sequence analysis revealed that nUSP<sub>2149</sub> contains an unusual 3' end that is not homologous to published USP sequences, therefore additional 3' sequence was determined as follows. Sense primer USP12-5I, having the nucleotide sequence 5' CTG CAT AAA ATG CCT AAA GTC GCG GAC 3', designated herein as SEQ ID NO:57, was used in combination with antisense primer USP11-3I, SEQ ID NO:56, to produce a PCR product using 5 µl of a 1:50 dilution of the flea head and nerve cord RACE cDNA pool described in Example 1 under standard PCR conditions. The resulting PCR product was a fragment of about 1975 base pairs, denoted herein as nUSP<sub>1975</sub>. The PCR product was purified using Qiagen's Qiaquick™ kit and cloned into the pCRII TA™ vector. Clones were prepared using a Biorad Quantum™ mini prep kit and the manufacturer's protocol, available from Biorad, Hercules, CA, and preferred clones were identified by restriction enzyme digest using 20 U/ µl *Eco* RI. One clone was isolated and sequenced using TA+ and TA- primers. The resulting nucleic acid sequence of nUSP<sub>1975</sub> has a coding strand presented herein as SEQ ID NO:32 and a complementary strand presented herein as SEQ ID NO:34.

[0162] Translation of SEQ ID NO:32 suggests that nucleic acid molecule nUSP<sub>1975</sub> encodes a full-length USP protein of 474 amino acids, referred to herein as PUSP<sub>474</sub>, having an amino acid sequence represented by SEQ ID NO:33, assuming the initiation

codon spans from nucleotide 454 through nucleotide 456 of SEQ ID NO:32 and the termination codon spans from nucleotide 1876 through nucleotide 1878 of SEQ ID NO:32. The coding region encoding PUSP<sub>474</sub>, is represented by nucleic acid molecule nUSP<sub>1422</sub>, having a coding strand with the nucleic acid sequence represented by SEQ ID NO:35 and a complementary strand with nucleic acid sequence represented by SEQ ID NO:37. The amino acid sequence of PUSP<sub>474</sub> predicts that PUSP<sub>474</sub> has an estimated molecular weight of about 52 kDa and an estimated pI of about 8.4. A DNA probe comprising nucleotide 99 through nucleotide 1878 of SEQ ID NO:32 was labeled with <sup>32</sup>P and used to probe separate samples of *C. felis* genomic DNA which had been digested with *Eco* RI and *Eco* RV, respectively. One to three bands of digested DNA hybridized with labeled probes, using standard USP hybridization conditions described herein, indicating that each of these genes are single copy number in genes.

- [0163] Comparison of amino acid sequence SEQ ID NO:33 with amino acid sequences reported in GenBank indicates that SEQ ID NO:33 showed the most homology, i.e., about 56% identity between SEQ ID NO:33 and a *Drosophila melanogaster* steroid hormone receptor-like protein, GenBank Accession No. S13119. Comparison of SEQ ID NO:35 with nucleic acid sequences reported in GenBank indicates that SEQ ID NO:35 showed the most homology, i.e., about 51% identity between SEQ ID NO:35 and a nucleic acid molecule encoding a *Drosophila melanogaster* steroid hormone receptor-like protein, GenBank Accession No. X52591. A comparison of nUSP<sub>1749</sub> and nUSP<sub>1975</sub> indicates that these molecules represent different variants of USP in *C. felis*. Percent identity calculations were performed using GCG version 9.0-UNIX using default parameters.

#### Example 4

- [0164] This example describes the expression of *C. felis* EcR and USP proteins.
- [0165] *A. EcR Expression* A putative ligand binding site of *C. felis* EcR spanning nucleotide 1549 to nucleotide 2161 of SEQ ID NO:5, referred to herein as nECR<sub>612</sub>, having a coding strand with the nucleic acid sequence represented by SEQ ID NO:38 and a complementary strand with nucleic acid sequence represented by SEQ ID NO:39, was isolated and expressed as follows. Primer EcR-LBD-F, having nucleotide sequence

5' GCG **GGA TCC** CAA GAT GGA TAT GAA CAA CCT 3', designated herein as SEQ ID NO:58 and having a *Bam* HI site indicated in bold, was used in combination with antisense primer EcR-LBD-R, having nucleotide sequence 5' GCG **GAA TTCTCA** ATC CCA AAT TTC TTC TAA AAA TCT 3', designated herein as SEQ ID NO:59 and having an *Eco* RI site indicated in bold, to produce a PCR product under standard PCR conditions using nECR<sub>2822</sub> as the template. The resulting PCR product was cut with 20 (U/  $\mu$  l) each of *Eco* RI and *Bam* HI restriction endonucleases, and subcloned into pGEX-6P1 expression vector, available from Pharmacia, Piscataway, NJ, which had been cut with *Eco* RI and *Bam* HI. The resulting recombinant molecule, referred to herein as pGEX-nECR<sub>612</sub>, was transformed into *E. coli* strain BL21, available from Novagen, Madison, WI, to form recombinant cell *E. coli*:pGEX-nECR<sub>612</sub>. Colonies were screened by restriction enzyme digestion with 20 U/  $\mu$  l each of *Bam* HI and *Eco* RI after DNA was isolated using the Qiaspin™ Mini Prep kit. Preferred colonies were then incubated in the presence of 1 mM isopropylthio-  $\beta$  -galactoside (IPTG) to induce expression of recombinant protein. Expression of protein was confirmed using antibodies that bind to the GST tag and Western Blot analysis which showed expression of an about 55 kD protein.

[0166]

*B. USP Expression* A putative ligand binding site of *C. felis* USP spanning nucleotide 857 to nucleotide 1633 of SEQ ID NO:26, referred to herein as nUSP<sub>776</sub>, having a coding strand with the nucleic acid sequence represented by SEQ ID NO:40 and a complementary strand with nucleic acid sequence represented by SEQ ID NO:41, was isolated and expressed as follows. Primer USP-LBD-F, having nucleotide sequence 5' GCG **GGA TCC** CTC TGT TCG AGA TTT AAC GGT A 3', designated herein as SEQ ID NO:60 and having a *Bam* HI site indicated in bold, was used in combination with antisense primer USP-LBD-R, having nucleotide sequence 5' GCG **AAG CTTTCA** ACC GAT GGG TCC GCC 3', designated herein as SEQ ID NO:61 and having a *Hind* III site indicated in bold, to produce a PCR product under standard PCR conditions using nUSP<sub>1749</sub> as the template. The resulting PCR product was cut with 20 U/  $\mu$  l each of *Bam* HI and *Hind* III restriction endonucleases, and subcloned into the pTrc-His-B expression vector, available from Invitrogen, which had been cut with *Bam* HI and *Hind* III. The resulting recombinant molecule, referred to herein as pTrc-His-nUSP<sub>776</sub> was transformed into *E. coli* strain BL21 to form recombinant cell *E. coli*:pTrc-nUSP

718 Colonies were screened by restriction enzyme digestion with 20 U/  $\mu$ l each of *Bam* HI and *Hind* III after DNA was isolated using the Qiaspin™ Mini Prep kit. Preferred colonies were then incubated in the presence of 1 mM IPTG to induce expression of recombinant protein. Expression of protein was confirmed using antibodies that bind to the T7 tag and Western Blot analysis which showed expression of an about 36 kD protein.

[0167] *C. EcR and USP co-expression* The ligand binding sites of *C. felis* EcR and USP described in Example 3A and 3B were co-expressed as follows. The recombinant molecule pTrc-His-nUSP<sub>776</sub> was used as the template in a PCR reaction using sense primer USP-GEX-LBD-F, having nucleotide sequence 5' GCG **CCC GGG** GGA TTA ACT TTA TTA TTA AAA ATT AAA 3', designated herein as SEQ ID NO:62 and having an *Xma* I site indicated in bold, and antisense primer USP-GEX-LBD-R, having nucleotide sequence 5' GCG C **GC GGC CGC AAG CTT** TCA ACC GAT GGG TCC 3', designated herein as SEQ ID NO:63 and having a *Not* I site indicated in bold. PCR reactions were performed using the following conditions: (1) one cycle at 95 ° C for two minutes and forty seconds; (2) thirty-five cycles at 95 ° C for thirty seconds, 52 ° C for thirty seconds, and 72 ° C for one minute and thirty seconds; and (3) one cycle at 72 ° C for seven minutes, in reactions containing 1.5 mM MgCL<sub>2</sub>, 0.2 mM dNTPs, 1  $\mu$  M of each primer, 1  $\mu$ l of 5 U/  $\mu$ l *Taq* polymerase, and 1  $\mu$ l of template. The resulting PCR product was a fragment of about 943 base pairs containing the ribosome binding site of pTrc-His and the ligand binding site of USP, designated herein as nUSP<sub>943</sub>, having a coding strand with the nucleic acid sequence represented by SEQ ID NO:42 and a complementary strand with nucleic acid sequence represented by SEQ ID NO:43.

[0168] A dicistronic vector containing the ligand binding sites of USP and EcR was produced as follows. The recombinant molecule pGEX-nECR<sub>612</sub> and the PCR product nUSP<sub>943</sub>, each were digested with 10 U/  $\mu$ l of *Xma* I and *Not* I restriction endonucleases, available from New England Biolabs. The two restriction enzyme digested products were combined and allowed to ligate to form a recombinant molecule designated pGEX-EcR<sub>612</sub>-USP<sub>943</sub>, which was transformed into *E. coli* strain BL21 to form the recombinant cell referred to as *E. coli*:pGEX-EcR<sub>612</sub>-USP<sub>943</sub>.

[0169] Colonies were screened by restriction enzyme digestion with 20 U/  $\mu$ l each of *Bam* HI and *Not* I after DNA was isolated using the Qiaspin <sup>TM</sup> Mini Prep kit. Selected colonies were then incubated in the presence of 1 mM IPTG to induce expression of recombinant protein. Expression of the recombinant proteins was confirmed by Western Blot analysis using antibodies that bind specifically to the T7 tag and the GST tag of the recombinant proteins. The resulting Western identified an about 55 kD protein and an about 36 kD protein.

[0170] While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims: